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Identification of the prion protein Doppel in spermatozoa and its impact in fertility

Leonor Matias Ferreira

DISSERTAÇÃO

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Orientada por:

Doutora Rosa Lino Neto Pereira – Unidade Estratégica de Investigação e Serviços em Biotecnologia e Recursos Genéticos, Instituto Nacional de Investigação Agrária e Veterinária, Santarém

Profª Doutora Deodália Dias – Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa

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SUMÁRIO

Identificação da proteína tipo-priónica Doppel em espermatozóides e o seu impacto na fertilidade

O gene *PRNP* é responsável pela expressão da proteína priónica celular (PrP^{C}), que, após sofrer uma conversão pós-tradução numa isoforma anormal e insolúvel (PrP^{Sc}), está associada à transmissão e à patogénese das encefalopatias espongiformes transmissíveis (EETs). Mais recentemente descobriu-se um outro gene, o *PRND*, que se julga ter resultado da duplicação ancestral do *PRNP*. O *PRND* é responsável pela expressão de uma proteína tipo priónica Doppel, que ao contrário da PrP^{Sc}, não está associada à propagação de EETs. A proteína Doppel é pouco expressa no cérebro, contrariamente à PrP^{C}, mas é expressa no coração e, principalmente, nas células germinativas e de Sertoli (ratinho, ratazana, suínos, humanos, ovinos e bovinos), nas células de Leydig (caprinos), nas células epiteliais do epidídimo (javali, suínos), e nos folículos ováricos (células da granulosa). Apesar da expressão do gene *PRND* aparentemente ser constante nas células de Sertoli, a sua expressão ao nível das células germinativas masculinas parece variar bastante. Como tal, diferentes padrões de expressão deste gene podem estar associados não só à espécie, mas também à fertilidade ou infertilidade do macho. Por outro lado, os ratinhos fêmeas *PRND-knockout,* sem expressão de Doppel, são viáveis e férteis, mas os machos são estéreis, o que sugere que esta proteína possa ter um papel importante na gametogénese e fertilidade masculina. Os machos *PRND-knockout* têm um comportamento sexual normal, com concentrações de espermatozóides próximas das normais, mas sem capacidade de realizar a reacção acrossómica e de fertilizar os oócitos (não penetram na zona pelúcida). Estudos recentes demonstram que a adição da proteína Doppel ao meio de capacitação aumenta a capacidade de fertilização *in vitro* de espermatozóides ovinos, reforçando a hipótese desta proteína ter funções ao nível da regulação da função acrossómica e da fertilidade masculina. Resultados anteriores evidenciaram uma associação entre os polimorfismos do gene *PRND* ovino e a capacidade de fertilização e de crio-resistência dos espermatozóides. No entanto, a função específica desta proteína permanece por esclarecer.

Este trabalho tem como objectivo identificar polimorfismos no gene *PRND* em indivíduos do sexo masculino (humanos e ovinos) e comparar a qualidade e crio-resistência dos seus espermatozóides com base nos diferentes polimorfismos encontrados (humano: codão 174 e 3'UTR; ovino: codão 26) através da análise da motilidade, vitalidade, concentração e morfologia de sémen fresco e submetido à criopreservação. O sémen ovino crio-preservado também foi avaliado quanto ao potencial mitocondrial transmembranar de espermatozóides pós-*swim-up*, quanto ao seu estadio de capacitação e quanto à sua capacidade de fertilização *in vitro*. A expressão de Doppel ovino em espermatozóides da raça Churra Galega Mirandesa também foi caracterizada e quantificada de acordo com o genótipo do codão 26 do

gene *PRND* através de imunofluorescência indirecta e de citometria de fluxo, usando anticorpos monoclonais produzidos especificamente contra este péptido.

Os resultados deste trabalho permitiram, pela primeira vez, detectar na população portuguesa três polimorfismos do gene *PRND* humano (dois polimorfismos no codão 174 e um 38 pares de bases 3'UTR), bem como o aumento da viabilidade do sémen após a descongelação em dadores com o genótipo CT do codão 174 (genótipo *wild-type*) associado ao genótipo TT do polimorfismo 3'UTR (genótipo *wild-type*) deste gene. Para além disto, é possível concluir que a presença do genótipo AA do codão 26 do gene *PRND* ovino melhora a crio-resistência do sémen e a produção de embriões. Em ovinos, estes resultados não só permitiram identificar a proteína Doppel em sémen fresco, através de imunofluorescência indirecta, como também permitiram a detectar, por citometria de fluxo, uma diminuição da expressão desta proteína em espermatozóides ovinos submetidos ao processo de criopreservação .

Este trabalho permitiu ainda concluir que a identificação da proteína Doppel ovina em espermatozóides frescos, bem como a diminuição da sua expressão após o processo de criopreservação, revelam uma importante função fisiológica desta proteína na fertilidade masculina, abrindo novos horizontes na selecção de machos com melhor crio-resistência e capacidade reprodutiva, ou no desenvolvimento de tratamentos clínicos de infertilidade.

Palavras-chave: proteína tipo priónica Doppel, espermatozóides, fertilidade, polimorfismo.

ABSTRACT

Identification of the prion-like Doppel protein in spermatozoa and its impact in fertility

The *PRNP* (prion protein gene) is responsible for the expression of the cellular prion protein (PrP^{C}). After undergoing a post-translation conversion into an abnormal isoform, scrapie associated PrP (PrP^{Sc}) is involved in the transmission and pathogenesis of transmissible spongiform encephalopathies (TSE), including scrapie in sheep. More recently, Moore *et al.* (1999) discovered another gene whose locus is located downstream of that of the *PRNP* and named it *PRND* (prion-like protein gene). Also, *PRND* gene is thought to emerge from early gene duplication. This new gene is responsible for the expression of the downstream prion like protein, also known as Doppel or Dpl, which is not involved in prion propagation. Doppel is highly expressed in testis (Sertoli and germ cells), in the heart, in the ovaries (granulosa cells) and, unlike PrP^{C}, is poorly expressed in the Central Nervous System. In *PRND-knockout* mice, females are fertile and viable, while males are infertile, suggesting a major role in male reproduction. Several studies have shown that the lack of Doppel can be associated with deficient acrosomic reaction and malformations on spermatozoa head and middle piece, impeding the spermatozoa to penetrate in the zona pellucida. Recent studies from our research group have demonstrated that capacitation medium supplemented with Doppel protein increases fertilization ability in sheep. Moreover, previous results showed an association between ram *PRND* gene polymorphisms and sperm fertilization ability and cryoresistance. However, the accurate physiological function of Doppel remains unclear. This work aimed to identify, characterize and quantify the expression of Doppel protein in ovine spermatozoa with different *PRND* polymorphisms by indirect immunofluorescence and flow cytometry. Moreover, the quality and cryoresistance of human and ovine sperm were compared based on different *PRND* polymorphisms, by analyzing the motility, vitality, concentration and morphology of fresh and thawed sperm. Ram semen was also evaluated by analyzing mitochondrial membrane potential of post-swim-up spermatozoa and by testing its in vitro fertilization ability. In conclusion, our results confirm that the AA genotype in codon 26 of ovine *PRND* gene is important for an improved semen cryoresistance and embryo production. Also, three human *PRND* polymorphisms were detected for the first time in male Portuguese population and an enhanced viability after thawing and centrifugation in density gradients of post-thawed sperm observed in male donors carrying CT genotype of codon 174 polymorphisms linked to TT genotype of 3'UTR of human *PRND* gene. Furthermore, the identification of Doppel protein in ovine semen and its decrease expression after the frozen-thawed process strongly suggests an important physiological function in male fertility.

Keywords: prion-like protein Doppel, spermatozoa, fertility, polymorphism.

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LIST OF ABREVIATIONS

Δψmit	Inner mitochondrial membrane potential
µL	Microliter
µM	Micromolar
AP	Alkaline phosphatase
AR pattern	Acrosome-reacted spermatozoa
B pattern	Capacitated with intact acrosome spermatozoa
BME	Basal Medium Eagle
BP	Band pass filter
bp	Base pair
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
CNS	Central nervous system
COC	Cumulus oocyte complexes
Concent	Spz concentration
CpG	Cytosine-phosphate-guanine
CTC	Chlortetracycline
^{Ctm}PrP	C-transmembrane form of PrP
CWD	Chronic wasting disease
DABCO	1,4-Diazabicycol (2.2.2) octane
DAPI	4,6-diamidino-2-phenylindole, dihydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
Dpl	Doppel protein
EC	European Community
eCG	Equine chorionic gonadotropin
ELISA	Enzyme-linked immunosorbent assay
ERK1/2 MAP kinases	Extracellular signal-regulated mitogen-activated protein kinase 1/2
F pattern	Non-capacitated spermatozoa
fCJD	Familial Creutzfeldt-Jakob disease
FCS	Foetal calf serum
FFI	Fatal familial insomnia
FITC	Fluorescein isothiocyanate

FSE	Feline spongiform encephalopathy
FT	Frozen-thawed semen
GPI	Glycosylphosphatidylinositol anchor
GSS	Gerstmann-Sträussler-Scheinker disease
HAT	Hypoxanthine-Aminopterin-thymidine media supplement
HEPES	4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid
HuDpl	Human Doppel
iCJD	Iatrogenic Creutzfeldt-Jakob disease
ICSI	Intra-cytoplasmic sperm injection
IIU	Intra-uterine sperm injection
IU	International unit
IVF	In vitro fertilization
JC-1	5,5',6,6'-tetra-chloro-1,1',3,3'-tetrathylbenzimidazolycarbocyanine iodide
LacZ	β-galactosidase gene
MEM	Minimum Essential Medium
Met	Methionine
MI	Individual motility
min	Minute
mL	Milliliter
mM	Millimolar
MRF-SSCP	Multiple restriction fragment – single-strand conformation polymorphism
mRNA	Messenger ribonucleic acid
NA	Numerical aperture
nm	Nanometer
nM	Nanomolar
^{Ntm}PrP	N-transmembrane form of PrP
ORF	Open reading frame
PBS	Dulbecco's Phosphate Buffered Saline
PCR	Polymerase chain reaction
PGF2α	Prostaglandin F2 α
PI	Propidium iodide
pmol	Picomole
PRN	Prion protein genomic locus
PRND	Prion-like Doppel protein gene
***PRND*$^{+}$**	Prion-like Doppel protein wild-type allele
***PRND*$^{+/+}$**	Prion-like Doppel protein gene

***PRND*$^{+/0}$**	Heterozygous prion-like Doppel protein gene
***PRND*^{0}**	Prion-like Doppel protein mutant allele
***PRND*$^{0/0}$**	Prion-like Doppel protein gene *Knockout*
PRNP	Prion protein gene
***PRNP*$^{0/0}$**	Prion protein gene *knockout*
PRNT	Prion protein testis-specific gene
PrP	Prion protein
PrP^{C}	Cellular prion protein isoform
PrP^{Sc}	Scrapie-associated prion protein isoform
Prt	Testis-specific prion-like protein
ROS	Reactive oxygen species
rpm	Revolutions per minute
s	Second
sCJD	Sporadic Creutzfeldt-Jakob disease
sFI	Sporadic fatal insomnia
SNP	Single nucleotide polymorphism
SOF	Synthetic oviduct fluid
SPRN	Shadow of prion protein gene
Spz	Spermatozoa
Thr	Threonine
TME	Transmissible mink encephalopathy
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
TSEs	Transmissible spongiform encephalopathies
T-TBS	Tris Buffered Saline with Tween® 20
UTR	Untranslated region
v/v	Volume per volume
vCJD	New variant Creutzfeldt-Jakob disease
Vh	Volt-hours
W	Watt
w/v	Weight per volume

1. INTRODUCTION

1.1. Historical approach to prion diseases and Doppel

The first prion disease in humans, named Creutzfeldt-Jakob disease (CJD), was described in 1929 by Creutzfeldt and Jakob, who depicted a progressive dementia associated with motion abnormalities, extensive vacuolation and astrocytic gliosis in the brain (Creutzleldt, 1920). Kuru was only discovered in the mid-1950s by Carlton Gajdusek who described it as an endemic disease among the Fore tribe, in New Guinea, and it was manifested as progressive gait ataxia in combination with abnormal behavior and a relatively rapid progression to death. This disease affected mostly women and children since during the cannibalistic feast the men could choose which body parts they wanted to eat, leaving the remains, including infectious brains, to the women and children (Hadlow, 1999; Mastrianni, 2004).

William Hadlow, a veterinarian-scientist, was the first to notice that Kuru and Scrapie, a transmissible disease of sheep, exhibited similar pathological features (Hadlow, 2008). This discovery led to success of transmission experiments of Kuru and CJD to chimpanzees, demonstrating they both carry the same spongiform pathology. Since this discovery, several other members of the family of transmissible spongiform encephalopathies (TSEs) of both mammals and humans have been discovered (Gajdusek *et al.*, 1966; Mastrianni, 2004). Later on, the discovery of new mechanisms for the origin and dissemination of infectious diseases, including prion diseases, granted Carlton Gajdusek a Nobel Prize in 1976 (Gajdusek, 1977; Mastrianni, 2004).

Until the mid-1990s, TSEs were considered to result from a 'slow virus' infection that could be inactive for years prior to manifestation of disease. However, neither viral particles nor nucleic acids were detected to support a viral theory (Prusiner, 1998). Instead, several evidences of an abnormal isoform of prion protein (PrP^{Sc}) appeared as the cause of TSEs, which granted Stanley Prusiner the second TSE-related Nobel Prize in 1997 for the discovery of an infectious particle responsible for transmission of TSEs: the prion protein (Prusiner, 1998). The scientific investigation of the transmission of prion protein diseases only began after the molecular genetic lesion had been identified. The *PRNP* gene, responsible for PrP^{C} expression, was firstly identified in families with prion disease by positional cloning or through the purification and sequencing of PrP^{C} in amyloid plaques, before transmissibility was verified (Prusiner *et al.*, 1998).

According to the 'protein-only' hypothesis, prions are infectious protein particles and they are the major contributing agents of TSEs in mammals. A hallmark of this type of pathology is the conversion of the normal cellular prion protein isoform (PrP^{C}), expressed by the *PRNP*, into a scrapie-associated isoform (PrP^{Sc}) with an abnormal conformation (Prusiner *et al.*, 1998). The etiology of TSEs can be sporadic, suggesting an inefficient degradation of PrP^{C} by the ubiquitin-proteasome system, dominantly

inherited, based in mutations within the *PRNP* gene that can lead to a spontaneous conversion of PrP^{C} to PrP^{Sc}, and acquired by infectious of previous contaminated tissues. The most common TSEs in humans and other mammals are briefly described in Table 1 (Prusiner *et al.*, 1998; Mastrianni, 2004; Fornai *et al.*, 2006; Colby and Prusiner, 2011).

Table 1 – Prion diseases in humans and other mammals (adapted from Mastrianni (2004) and Colby and Prusiner (2011))

Disease	Host	Etiology	Mechanism of pathogenesis
Kuru	Human	Acquired	Ritualistic cannibalism, consumption or direct contact with human brain infected with Kuru
iCJD	Human	Acquired	hGH, dura mater grafts, corneal transplants, β-HCG, neurosurgical procedures, medical equipment
vCJD	Human	Acquired	Consumption of contaminated bovine products
Scrapie	Sheep	Acquired	Infection
BSE	Cattle	Acquired or sporadic	Infection
TME	Mink	Acquired	Infection with sheep and cattle prions
CWD	Mule deer, elk	Acquired	Infection
FSE	Cat	Acquired	Infection with prion-contaminated bovine tissues or MBM
fCJD	Human	Inherited	Germline point mutations in *PRNP* gene
GSS	Human	Inherited	Germline point mutations in *PRNP* gene
FFI	Human	Inherited	Germline point mutations in *PRNP* gene
sCJD	Human	Sporadic	Somatic mutation, spontaneous conformation change from PrP^{C} to PrP^{Sc}
sFI	Human	Sporadic	Somatic mutation, spontaneous conformation change from PrP^{C} to PrP^{Sc}

iCJD – iatrogenic Creutzfeldt-Jakob disease; **vCJD** – new variant Creutzfeldt-Jakob disease; **BSE** – bovine spongiform encephalopathy; **TME** – transmissible mink encephalopathy; **CWD** – chronic wasting disease; **FSE** - feline spongiform encephalopathy; **fCJD** – familial Creutzfeldt-Jakob disease; **GSS** - Gerstmann-Sträussler-Scheinker disease; **FFI** – fatal familial insomnia; **sCJD** – sporadic Creutzfeldt-Jakob disease; **sFI** – sporadic fatal insomnia.

The classic TSE, also known as scrapie, has been observed in European sheep for more than 200 years, whereas bovine spongiform encephalopathy (BSE) in cattle seems to be associated with modern agricultural practices (1986). More recently, a new variant of CJD (vCJD) was transmitted to humans by ingestion of BSE-contaminated food products (Hil *et al.*, 1997; Scott *et al.*, 1999; Casalone *et al.*, 2004), meaning that the "species barrier" between cattle, sheep, human and other mammalian species was crossed by the biological properties of BSE, also known as Human Mad Cow disease, raising considerable concern for human health. Given this, the European Community (EC) started a selection program based on the known genotypes of *PRNP* that have been associated with different grades of susceptibility to scrapie. The identification of

4 different single nucleotide mutations in the *PRNP* coding region and their linkage to different resistance to scrapie allowed the elaboration of a "table of susceptibility", which includes 5 groups of grades of resistance (R1-R5), briefly described in Table 2. In 2003, the EC (decision nº 100/2003) implemented this selection program which aims to eradicate scrapie from its member states and to create disease-resistant animals, allowing the introduction of scrapie-resistant genes and eliminating the most susceptible ones, with the ram as a major selection target. With respect to *PRNP* (Table 2), three genotypes were not represented: ARR/ARH, ARH/ARH and VRQ/VRQ (Gama *et al.*, 2006). However, within small population, such strategy might drastically decrease valuable genetic diversity due to intensive selective breeding for disease-resistant genotypes (Ehling *et al.*, 2006; Gama *et al.*, 2006), leading to undesirable consequences which might include a blind selection for other genes linked to reproduction and/or to *PRNP*, like *PRND* (prion-like Doppel protein gene) and *PRNT* (prion protein testis-specific gene) (Lipsky *et al.*, 2008; Mesquita *et al.*, 2010).

Table 2 – Sheep *PRNP* polymorphisms in codon 136, 154 and 171 and the scrapie-associated susceptibility grades (adapted from Mesquita *et al.* (2010))

Susceptibility	**R1**	**R2**		**R3**			**R4**				**R5**	
***PRNP* genotype**	ARR	ARR	AHQ	ARR	AHQ	AHQ	ARQ	ARQ	ARR	AHQ	ARQ	ARH
	ARR	AHQ	AHQ	ARQ	ARQ	ARH	ARQ	ARH	VQR	VQR	VQR	VQR

R1 to R5: scrapie susceptibility groups (from less to more susceptible) corresponding to each *PRND* genotype.

Although *PRNP* was originally considered to be unique within the mammalian genome, it has been shown to have a paralogue: the downstream prion-like protein gene, also called prion-like Doppel protein gene (*PRND*). When *PRNP* was knocked out in mouse ($PRNP^{0/0}$), no phenotypical alterations were observed, supporting a non-critical function of *PRNP* or at least a redundancy of its function by another protein. Yet, ever since other lines of $PRNP^{0/0}$ have been created, displaying a distinct ataxic phenotype attributed not to the loss of function of PrP^{C} but to the deletion of variably lengthened segments of DNA, resulting in enhanced expression of a downstream protein. This protein was named Doppel (or Dpl) and is expressed by the *PRND* gene (Moore *et al.*, 1999). Latter on other family genes (*PRNT* and shadow of prion protein gene – *SPRN*) were identified, contributing together to the so called prion genetic complex (Premzl and Gamulin, 2007; Pimenta *et al.*, 2012b).

1.2. Prion genetic complex

The *PRN* genomic locus comprehends three genes: *PRND*, *PRNP* and *PRNT*, and they can be found on chromosomes 10 and 22, in sheep and human, respectively (Lampo *et al.*, 2007). The *SPRN* gene is not part of this genomic locus (Lampo *et al.*, 2007; Watts and Westaway, 2007; Pimenta *et al.*, 2011). *PRND* contributes, along with *PRNP*, *PRNT* and *SPRN* genes, to the "prion genetic complex". This four prion and prion-like genes show similar gene organizations, although they exhibit different expression patterns and have distinct biological functions (Premzl and Gamulin, 2007; Pimenta *et al.*, 2012b).

1.2.1. *PRNP*

The *PRNP* is a highly conserved housekeeping gene that has been described in fish (Premzl and Gamulin, 2007) and several eutherian species, like human, sheep, mouse (Lee *et al.*, 1998), bovine (Hills *et al.*, 2001) and hamster (Li and Bolton, 1997). *PRNP* gene has three exons in mouse, sheep (Lee *et al.*, 1998), rat (Saeki *et al.*, 1996) and bovine (Hills *et al.*, 2001), with the first two exons encoding the 5'UTR of mRNA, but only two exons in human (Lee *et al.*, 1998), with the first exon encoding the 5'UTR region. In placental mammals, *PRNP* promoters are all rich in cytosine-phosphate-guanine (CpG) islands and lack TATA box, despite some differences in gene structure and regulation of gene expression among species.

PRNP is expressed mostly within glia and neuronal synapses in the central nervous system (CNS), although it could be found in a broad range of vertebrate tissues, such as spleen, lymph nodes, lungs, heart, kidneys, muscle, mammary glands and both male and female gonads (Miranda *et al.*, 2011). This gene is clearly upregulated in the CNS at both fetal and adult stages, with PrP^{C} (Fig. 1) mainly localized to synapse membranes (Herms *et al.*, 1999). However, *PRNP* mRNA can also be found in adult epididymis at levels almost as high as in the brain (Makrinou *et al.*, 2002).

1.2.2. *SPRN*

The shadow of prion protein gene, also named *SPRN*, encodes the Shadoo protein and is located outside *PRN* genomic locus, more precisely in chromosomes 7, 10 and 22, in mice, humans and sheep, respectively (Lampo *et al.*, 2007; Watts and Westaway, 2007). It comprises two exons with the open reading frame (ORF) contained within a single exon, as *PRNP* and *PRND*. *SPRN* has been observed in eutherian and fish (Premzl *et al.*, 2003; Watts and Westaway, 2007).

In sheep, Shadoo expression presented high levels in cerebrum and in Purkinje cells of cerebellum, and low levels in testis, lymph node, jejunum, ileum, colon and rectum (Lampo *et al.*, 2007). In immunohistochemistry assays, Shadoo was immunopositive in the cerebrum, hippocampus, pituitary gland, medulla oblongata, thalamus and hypothalamus. Remarkably, a typical granular pattern was seen in most of the tested brain tissues, which might indicate that Shadoo is primarily expressed at synapses (Lampo *et al.*, 2007).

1.2.3. *PRNT*

The prion protein testis-specific gene (*PRNT*) is located 3 and 6 kb 3' downstream of *PRND* in human (Makrinou *et al.*, 2002) and cattle (Kocer *et al.*, 2007), respectively. This gene probably emerged due a duplication event during eutherian species divergence, since is present among other species. It has two exons in primates, bovine, horse and dog (Makrinou *et al.*, 2002; Harrison *et al.*, 2010). *PRNT* encodes the testis-specific prion protein-like (Prt).

PRNT gene promoters do not include CpG islands, which suggests a tissue-specific expression (Makrinou *et al.*, 2002; Premzl and Gamulin, 2007). *PRNT* expression in caprine and human tissues was studied only at mRNA level (Makrinou *et al.*, 2002; Kocer *et al.*, 2007). Recently, Pimenta *et al.* (2012b) were able to found ovine Prt, by indirect immunofluorescence, in sperm head apical ridge subdomain before and after in vitro capacitation, and by immunohistochemistry in spermatogonias, spermatocytes, spermatids and spermatozoa, indicating a function during ram spermatogenesis. Moreover, results showed that ovine Prt was found in seminiferous tubules, along the developing stages of germinal cells (spermatogonia, spermatocytes, spermatids and also in spermatozoa). Only caprine *PRNT* revealed a low and irregular expression pattern at different development stages, suggesting a different *PRNT* expression between species (Kocer *et al.*, 2007).

1.2.4. *PRND*

PRND is located 20 kb (humans; Makrinou *et al.*, 2002), 16.8 kb (sheep; Comincini *et al.*, 2001), 16 kb (mouse; Moore *et al.*, 1999) and 52 kb (cattle; Essalmani *et al.*, 2002). Doppel has a significant homology to the C-terminal segment of PrP^{C}, so it was proposed that *PRND* and *PRNP* arose by an early gene duplication event of an ancestral *PRN* gene (Moore *et al.*, 1999). It contains two exons in sheep, cattle (Comincini *et al.*, 2001) and goat (Uboldi *et al.*, 2005), and three exons in mouse and human (Moore *et al.*, 1999).

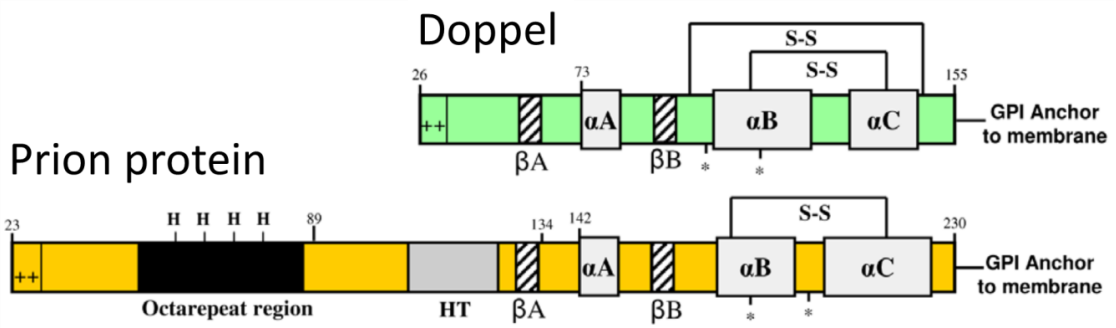

Fig. 1 – Murine Doppel and PrP^C secondary structure elements (adapted from Pimenta (2013))

PRND gene presents characteristics of a housekeeping gene, though Doppel is mainly expressed in male genital tract, suggesting that *PRND* transcription and/or translation is probably submitted to a tight regulation (Comincini *et al.*, 2001). *PRND* mRNA was found to be expressed in the spleen, suggesting a potential role for Doppel in lymphoid cells (Li *et al.*, 2000); however, the immune system appeared to be normally developed in $PRND^{0/0}$ mice (Behrens *et al.*, 2002). Doppel can be found in both Sertoli and germ cells in mice, rat, swine (Behrens *et al.*, 2002; Serres *et al.*, 2006), humans (Peoc'h *et al.*, 2002), ovine (Espenes *et al.*, 2005) and bovine (Rondena *et al.*, 2005), in goat Leydig cells (Kocer *et al.*, 2007) and in boar epididymis epithelial cells (Serres *et al.*, 2006), which allied to the sterility presented in $PRND^{0/0}$ mice (Behrens *et al.*, 2002) suggests an important physiological role on male fertility (Moore *et al.*, 1999; Silverman *et al.*, 2000; Tranulis *et al.*, 2001; Peoc'h *et al.*, 2002; Rondena *et al.*, 2005; Serres *et al.*, 2006; Kocer *et al.*, 2007). Doppel is permanently expressed in Sertoli cells, but its expression in the germ cells can vary accordingly to species. In ovine, Doppel can be detected in the seminiferous epithelium in the final stages of spermatogenesis, but it was not always detected in the ejaculated spermatozoa. On the other hand, bovine and human Doppel was found to be expressed in the ejaculated spermatozoa (Espenes *et al.*, 2005; Rondena *et al.*, 2005; Serres *et al.*, 2006; Kocer *et al.*, 2007). This may suggest an expression level in ovine smaller than the detection limit of the methods used so far.

1.3. Prion and Prion-like proteins: structural and biochemical properties

1.3.1. PrP^C

The cellular prion protein (PrP^C) is a non-pathogenic glycoprotein attached to the cell surface by a GPI anchor that is translated as a 253 and 256 amino acid protein in human and sheep, respectively. The PrP^C has a long and flexible N-terminal tail, three α-helices and two stranded antiparallel β-sheet (Mo *et al.*, 2001). This protein can present at least three distinct topological orientations: the fully extracellular (PrP^C) form (Hölscher *et al.*, 2001), and two transmembrane isoforms with opposite sequence orientations (^{Ntm}PrP and ^{Ctm}PrP) (Hegde *et al.*, 1998; Nicolas *et al.*, 2009).

The normal physiological function of PrP^C remains uncertain, although there is some evidence that it may have a protective effect against neuronal damages. More precisely, PrP^C is upregulated following ischemic brain damage, in both human and mice (McLennan *et al.*, 2004; Weise *et al.*, 2004), and has a neuroprotective activity against apoptosis in vivo (Nishida *et al.*, 1999; Moore *et al.*, 2001; Rossi *et al.*, 2001; Westergard *et al.*, 2007). Another hypothesis is that PrP^C could also be a signal transduction protein since it activates in vitro a signaling pathway and targets the MAP kinases ERK1/2 of both neuronal and non-neuronal cells.

The scrapie-associated prion protein isoform (PrP^{Sc}) is derived from the PrP^C by a posttranslational process involving a profound conformation change. Although these two isoforms share the same amino acid sequence, their biochemical and biophysical properties are remarkably different. PrP^C is predominantly α-helical (Mo *et al.*, 2001), soluble in non-denaturing detergents and completely sensitive do proteinase-K digestion. On the other hand, PrP^{Sc} biochemical structure has more than 40% of β-sheet, is insoluble in non-denaturing detergents, and has a relative resistance to proteinase-K (Mastrianni, 2004).

1.3.2. Shadoo

Shadoo (the Japanese word for shadow) is a hypothetical GPI-anchored protein that exhibits homology to the N-terminus of PrP^C. The expression of this prion-like protein stimulates a PrP^C-like neuroprotective activity and several other biochemical and cell biological properties also exhibited by PrP^C, although is low in cerebellar granular neurons containing PrP^C, high in PrP^C-deficient dendritic processes and absent during prion infections, suggesting an interference of PrP^{Sc} in the physiological protective activity of Shadoo (Watts *et al.*, 2007).

1.3.3. Prt

Human Prt and PrP^C only have 44% similarity and 30% identity, whereas human *PRND* and *PRNT* only have 50% similarity and 42% identity. In bovine, *PRNT* encodes a 55 amino acid N-terminally truncated protein and has only 55% identity to its human counterpart. No signal peptides were predicted for Prt, suggesting that Prt could be an intracellular protein (Kocer *et al.*, 2007; Premzl and Gamulin, 2007). The accurate biological function of this prion protein-like remains unclear, although Pimenta *et al.* (2012b) and Pimenta (2013) suggests a role for Prt protein in the ram reproductive physiology, namely in the fertilization process.

1.3.4. Doppel

As referred, Doppel was firstly recognized due to an ataxic phenotype of a $PRNP^{0/0}$ mice line, and direct expression of Doppel in $PRNP^{0/0}$ mice have been shown to produce ataxia and loss of Purkinje cells at 6-12 months of age. However, no pathological characteristics of prion diseases were observed. Even in neuronal tissues of $PRNP^{0/0}$ mice, the absence of Doppel does not affect disease progression of experimental prion pathogenesis (Behrens *et al.*, 2001; Moore *et al.*, 2001). There is some evidence suggesting that Doppel may be toxic to neurons, but no mutations of *PRND* have yet been linked to any inherited prion disease, nor there is evidence of high expression of Doppel in patients with PrP-induced diseases (Mead *et al.*, 2000; Behrens *et al.*, 2001; Cui *et al.*, 2003). Like PrP^C, Doppel is a GPI anchored glycoprotein structured by a 3 α-helices and 2 β-sheets, although it has only 25% amino acid similarity to PrP^C and lacks the distinctive PrP^C repeats and the hydrophobic domain (Silverman *et al.*, 2000).

1.4. Prion and prion-like proteins: impact in fertility

1.4.1. PrP^C

In situ hybridization analysis indicates that *PRNP* mRNA in adult testis can be elevated to levels almost as high as in the brain, showing positive signals in spermatogenic cells, but not in somatic cells (Sertoli and Leydig cells) (Makrinou *et al.*, 2002; Schneider *et al.*, 2003). The signals were observed moderately in spermatogonia and strongly in spermatocytes and round spermatids, but not in elongated spermatids and spermatozoa, indicating that PrP^C may have a possible role in germ cell differentiation during mammal spermatogenesis (Fujisawa *et al.*, 2004). PrP^C can also have an important function in regulation of spermatogenesis oxidative stress, as a cooper binding protein (Brown *et al.*, 1997a and b). In the ovine reproductive tract, Gatti *et al.* (2002) showed that different PrP^C isoforms were present in the male genital tract fluids. Moreover these isoforms were secreted by the epididymal epithelium.

1.4.2. Shadoo

Young *et al.* (2011) created a mouse lineage carrying a transgenic *SPRN* (*SPRN*-LacZ) and was able to verify that Shadoo was expressed in Leydig cells, suggesting an important function in the development of the male reproductive system, and in the granulosa cells of the developing follicle, reinforcing the previous hypothesis of an association between *SPRN* and fertility, demanding further investigation.

1.4.3. Prt

Prior to the studies of Pimenta *et al.* (2012b), Prt expression pattern was studied only at mRNA level in human, caprine and ovine (Makrinou *et al.*, 2002; Kocer *et al.*, 2007). The three human Prt isoforms were only detected in adult testis, more precisely in Sertoli, Leydig and germ cells, revealing an important role in sperm production (Makrinou *et al.*, 2002). Also, Prt isoforms were not detected in any of the human fetal tissues analyzed (Makrinou *et al.*, 2002). This results indicate that Prt function is only required at a post-pubertal stage, when the above-mentioned cell types are actively involved in the production of male sex hormones and sperm. More recently, Pimenta *et al.* (2012b), based on immunofluorescence and immunohistochemistry assays, were able to report for the first time the location of ovine Prt in spermatozoa, confirming that ovine *PRNT* gene is effectively translated. Furthermore Pimenta *et al.* (2013a) showed that by blocking Prt function through the addition of an anti-Prt polyclonal antibody the fertilization rate was decreased. Taken together, these observations suggest that Prt could exert its main function in the fertilization process, potentially in the initial steps due to its location in the acrosome of ovine spermatozoa.

1.4.4. Doppel

Behrens *et al.* (2002) created homozygous mutant mice lacking Doppel (*PRND*$^{0/0}$) from intercrossed heterozygous mice carrying the mutated *PRND* allele (*PRND*$^{+/0}$) and verify that both male and female *PRND*$^{0/0}$ mice displayed normal growth and survival, demonstrating the absence of obvious detrimental effects of Doppel deficiency on development and growth. Doppel was present in the testis of *PRND*$^{+/+}$ and *PRND*$^{+/0}$, but it was absent in homozygous mutants. Summarizing, *PRND*$^{0/0}$ females appear to be viable and fertile, and *PRND*$^{0/0}$ males were infertile (Behrens *et al.*, 2002; Peoc'h *et al.*, 2002; Paisley *et al.*, 2004; Espenes *et al.*, 2005).

Although *PRND*$^{0/0}$ male mice presented normal sexual behavior with normal number of copulation plugs and reduced or normal sperm concentrations, the sperm showed malformed spermatids and appeared to be unable to undergo the normal acrosome reaction, resulting in the inability to penetrate the zona pellucida of the oocyte (Behrens *et al.*, 2002; Paisley *et al.*, 2004). Yet, partially mechanical dissection of zona pellucida could restore in vitro fertilization ability. Since Doppel is a highly glycosylated protein (Moore *et al.*, 1999; Silverman *et al.*, 2000), and oligosaccharides have been implicated in signaling for the acrosome reaction (Wassarman and Litscher, 2001), it is plausible that Doppel could be directly involved in sperm-egg interaction (Behrens *et al.*, 2002).

The number of spermatozoa in the cauda epididymis of *PRND*$^{0/0}$ males was reduced in 50%, when compared to wild-type controls. Also the motility of mutant sperm was significantly decreased, suggesting a spermatogenesis defect (Behrens *et al.*, 2002). In sheep, Espenes *et al.*, (2005) detected the transient presence of Doppel in the final stages of spermiogenesis, which points to an important role of this protein in the final remodeling of spermatids prior to their release into the seminiferous lumen. Although Doppel was not detected in the seminiferous tubules of *PRND*$^{0/0}$ mice, histological examination revealed normal distribution and numbers of spermatogonia and spermatocytes. However, the transformation of round spermatids into testicular spermatozoa was abnormal in Doppel-deficient testis. Moreover, the expression of maturation stage-specific genes in testis appears to be normal, despite the absence of Doppel (Behrens *et al.*, 2002).

Serres *et al.* (2006) hypothesized that Doppel could be acquired during the passage of maturing spermatozoa through the epididymis, as it has been described for other GPI proteins anchored to spermatozoa, suggesting a possible epididymis origin of Doppel. Interestingly, haploid spermatozoa lacking Doppel gene (*PRND*^{0}) are perfectly fertile when generated in the context of a heterozygous (*PRND*$^{+/0}$) mouse. This might due to the fact that sperms spends much of the maturation time in the form of syncytia where all maturating cells are connected to each other by cytoplasmic bridges, allowing Doppel protein to be transferred from *PRND*$^{+}$ to *PRND*^{0} spermatids and rescue fertility (Behrens *et al.*, 2002). Pimenta *et al.* (2012a) recently demonstrated that ovine

sperm supplementation with recombinant Doppel protein during in vitro capacitation significantly improved spermatozoa motility, vigor, viability and fertilization rate, suggesting a major role in ovine sperm capacitation and in the consequent fertilization process.

Behrens *et al.* (2002) concluded that Doppel (Fig. 2) deficiency did not interfere with mice embryonic and postnatal development. However, Paisley *et al.* (2004) used different *PRND*$^{0/0}$ mouse lines with a different genetic background and demonstrated that Doppel can also be required for sperm to contribute to embryonic development beyond the morulae stage. Also, high levels of damaged DNA were observed, which indicates a possible involvement in protection from oxidative stress. In fact, it has been demonstrated that ROS can modulate fertility: increasing ROS can reduce sperm motility and performance (Whittington *et al.*, 1999).

A possible association between *PRND* gene polymorphisms and ram sperm traits, freezability and fertility was recently suggested by Baptista *et al.* (2008) and *Pereira et al.* (2009). A multiple restriction fragment – single-strand conformation polymorphism (MRF-SSCP) analysis of genomic DNA of some Portuguese ovine breeds allowed the identification of a polymorphism in codon 26 of *PRND* gene. A synonymous substitution G→A was revealed and gave rise to three genotypes: GG (wild-type homozygous), GA (mutant heterozygous) and AA (rare mutant homozygous). Although AA genotype rams were not analyzed, the results showed an improved concentration for the ovine semen carrying the GA genotype, over GG genotype, as well as an improvement in sperm fresh vitality and a decrease of spermatozoa head anomalies after thawing. Despite being a synonymous substitution, it can affect protein folding and its activity/substrate specificity (Kimchi-Sarfaty *et al.*, 2007), and the presence of the allele A in codon 26 genotype might improve fertility in GA animals. In human, Peoc'h *et al.* (2002) investigated whether the polymorphism at codons 26 (T26M), 56 (P56L) and 174 (T174M) of human *PRND* would affect the processing the Doppel protein. There were no major abnormalities in cellular trafficking of the human Doppel (HuDpl; Fig. 2) and no cell toxicity effect resulting from each polymorphism in comparison to the wild-type HuDpl. However, it remains unclear whether these polymorphisms could modify structural and/or functional aspects of Doppel, affecting spermatogenesis and/or spermatozoa motility.

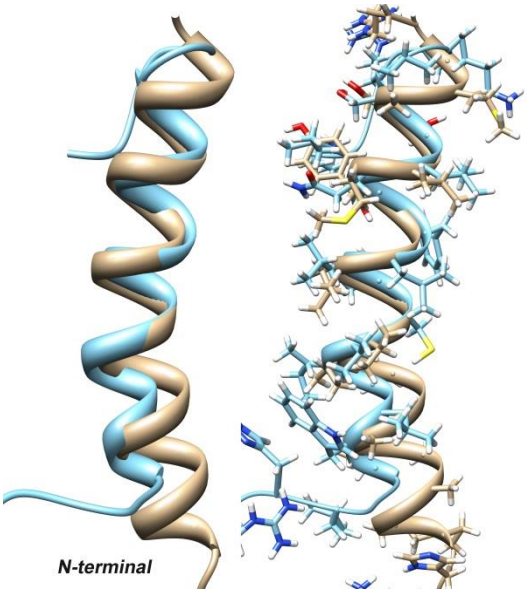

Fig. 2 – Comparison between ovine (blue) and human (brown) Doppel tertiary structures (adapted from Pimenta (2013))

1.5. Objectives

This work aimed to identify polymorphisms in *PRND* gene of male individuals (human and rams) and compare the quality and cryoresistance of their sperm based on those identified polymorphisms (human: in codon 174 and 3'UTR; ovine: codon 26), by analyzing the motility, vitality, concentration and morphology of fresh and frozen-thawed semen. Ram semen quality and cryoresistance was also evaluated by analyzing mitochondrial membrane potential of post-swim-up semen and by testing its in vitro fertilization ability. Additionally, ovine Doppel expression was characterized and quantified in semen from Churra Galega Mirandesa rams classified accordingly to codon 26 genotype of *PRND* gene. This evaluation was performed by indirect immunofluorescence and by flow cytometry using monoclonal antibodies produced specifically against this peptide.

2. MATERIALS AND METHODS

This work was carried out from September 2012 to April 2014 in the National Institute of Agriculture and Veterinary Research (INIAV, I.P., Santarém, Portugal), in close cooperation with the Center of Medical Reproduction of British Hospital Lisbon XXI, (Lisbon, Portugal) and with the Hygiene and Tropical Medicine Institute (IHMT, Lisbon, Portugal).

2.1. Production of monoclonal antibodies against ovine Doppel

2.1.1. Mouse immunization

The ovine 178 aa Doppel peptide (accession number NP_001009261, GenPept) was obtained from CASLO Laboratory Aps (Denmark) showing >95% purity, and used without further purification. Three five to six week-old female BALC/c mice were injected intraperitoneally with 60-80 μg of purified ovine Doppel emulsified in incomplete Freund's adjuvant (Sigma-Aldrich, USA) and boosted monthly (adapted from Pimenta *et al.* (2012b)). After 4 immunizations, blood samples were obtained from mice for serum titer determination by enzyme-linked immunosorbent assay (ELISA).

2.1.2. ELISA

Antibody responses generated against ovine Doppel were measured by ELISA (adapted from Pimenta *et al* (2012b)). Briefly, Costar 3690 96-well ELISA plate were coated with 0.1 μg/well or with 2 μg/well (ovine Doppel) overnight at 4^{o}C, followed by a four time wash and then blocked with 100 μL/well of 5% (w/v) skim milk (Difco, USA) in T-TBS (0.05% (v/v) Tween-20) for 1 h at 37^{o}C.

The ELISA plate was washed four times with T-TBS. Afterwards the plate was incubated with serum from immunized mice and from non-immunized control mouse for 1 h at 37^{o}C, followed by another four time wash with TBS-T.

Anti-mouse polyvalent immunoglobulin-alkaline phosphatase (AP) antibody (Sigma-Aldrich, USA), 100 μL/well of diluted 1:100, 1:500 and 1:1000 in PBS were added and incubated for 1 h at 37^{o}C. The p-Nitrophenyl Phosphate (p-NPP; J. T. Baker, The Netherlands) was added and incubated in the dark for 30 min at room temperature. Then the analysis of the AP activity was read on an ELISA plate reader (Dynex Technologies, USA) at an excitation wavelength of 405 nm and analyzed with Microplate Manager 4.0 software (Bio-Rad, USA).

2.1.3. Monoclonal screening for antibody producing hybridomas

Once the titer was high enough, cell fusion was performed. Three days before cell fusion, mice were boosted with ovine Doppel and then euthanized by cervical dislocation for spleen removal. Also, mice total blood was also collected in order to obtain antiserum.

Spleen cells were fused with myeloma cells Sp2/0-Ag14 during culture in DMEM (Sigma-Aldrich, USA) with HAT media supplement (Sigma-Aldrich, USA). Only hybrids between myeloma and spleen cells are capable of surviving in HAT supplemented medium.

At day 10, supernatants of wells containing hybridomas were tested by ELISA. Assay plate was prepared as described previously including a well with PBS (no antigen) to check for non-specific reactions of the primary antibody (antiserum). Four different controls were performed against the Doppel protein: a positive control with antiserum diluted 1:1000, and three negative controls (antiserum with no secondary antibody; no antiserum with secondary antibody; no antiserum or secondary antibody). Hybridoma supernatant was used as primary antibody.

The hybridomas selected by ELISA were expanded to 24-well plates and subsequently to 25 cm^2 tissue culture flasks. Supernatants (as primary antibody) were tested against by ELISA and also by Western Blotting. For storage, supernatants were filtered with a 0.22 µm syringe filter (Carl Roth GmbH + Co. KG, Germany) and kept at -20^{o}C.

2.2. Genetic analysis

In the present study, DNA samples from 10 rams from Churra Galega Mirandesa breed and from 29 men were analyzed for *PRND* polymorphisms screening.

2.2.1. DNA extraction and amplification

For the ram samples, DNA was extracted from blood using the Spin Protocol of DNA Purification Kit from Blood or Body Fluids (QIAmp®, Qiagen, Germany). The *PRND* coding region (exon 2) was amplified by PCR using specific primers (DOP1-F: 5'-TCCGACACAATGAGGAAACATCTGGG-3' and DOP1-R: 5'-TTGATCTCTGTGGCTGCCAACTTGC-3') designed based on the published ovine *PRND* gene sequence (accession number AF394223 GenBank) as in Pereira *et al.* (2009).

For the human samples, DNA was extracted from semen using an adapted protocol of Puregene DNA Purification Kit: DNA Purification Protocol for 50 µL Human Blood Strains (Puregene, Gentra Systems Inc., USA), using 100 µL of human semen and no glycogen. The human *PRND* coding region (accession number NM_012409.2, GenBank)

was amplified by PCR using specific primers (HuDpl72-F 5'-AGTTAACCCTGCACAACCCAA-3' and HuDpl73-R 5'-GCACCT TCAGAACACGCGT-3') as in Schröder *et al.* (2001).

PCR reactions were performed for both species in a UNOII thermocycler (Biometra, Germany) according to the following conditions: 50 ng of genomic DNA, 16 pmol of each primer, 1.25 U of NZYTaq DNA polymerase (Nzytech, Portugal), 1X reaction buffer (67mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20), 2 mM of $MgCl_2$, 200 µM of each dNTP, for a final volume of 25 µL. The amplification included an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C (ram samples) or 59°C (human samples) for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. The resulting amplification fragment of both ram (579 bp) and human (805 bp) was confirmed by electrophoresis on a 2% agarose gel containing 0.14 µg mL^{-1} of ethidium bromide (Qbiogene, USA), adapted from Pereira *et al.* (2009) and Schröder *et al.* (2001), respectively.

2.2.2. Polymorphism analysis by MRF-SSCP

For the ram samples, the amplified 579 bp fragment was analyzed by multiple restriction fragment – single-strand conformation polymorphism (MRF-SSCP): 7.5-9.0 µL of the amplification products were digested with 3 U of *EcoR* V restriction enzyme (Thermo Scientific, USA) at 37°C for 18h (Pereira *et al.*, 2009). For the human samples, the amplified 805 bp fragment were also analyzed by MRF-SSCP but with the following alterations: 5.0-10.0 µL of amplification products were digested with 3 U of *Bgl* I restriction enzyme (Metabion, Germany), adapted from Peoc'h *et al.* (2000). 22.5 µL of a denaturing solution (Table 17, Annex I) were added to the digestion products, for both species, as in Pereira *et al.* (2009).

The samples were heat-denatured at 95°C for 5 min, immediately chilled at 0°C and the total volume was loaded into a 13.6% polyacrylamide gel with 2.5% crosslinking. The polyacrylamide gel ran at 30 W for 4h22 (3000 Vh) at 20°C, and at 30 W for 4h23 (3000 Vh) at 15°C, for ram and human samples, respectively. Both polyacrylamide gel were ran in a DCode TM Universal Mutation Detection System (Bio-Rad, USA), coupled to a refrigeration system.

The resulting single-strand DNA band patterns were detected by silver staining using the PlusOne DNA Silver Staining Kit (Amersham Biosciences, UK). The ovine *PRND* genotypes were established by comparing the SSCP pattern obtaines for each samples with the ones characteristic of known *PRND* polymorphisms as in Pereira *et al.* (2009).

2.2.3. Sequencing

The sequencing reaction of PCR products from human samples containing the *PRND* gene was directly performed by capillary electrophoresis on an Abi 3130xl sequencer (Applied Biosystems, USA), using the same primers as above.

A bioinformatics analysis were performed by using a basic BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and DNA sequencing results were analyzed using a Sequence Scanner (v1.0, Applied Biosystems, USA) for polymorphism identification.

2.3. Semen collection and evaluation

2.3.1. Semen collection

Semen collection was conducted at the experimental farm of INIAV in compliance with the requirements of the European Union for farm animal's welfare and the Portuguese authority guidelines for animal experimentation. A total of five healthy and fertile rams from Churra Galega Mirandesa breed (*Ovis aries*), belonging to the Portuguese Bank of Animal Germplasm (BPGA) located at INIAV, were selected based on their *PRND* genotype (codon 26) – two donors with GG genotype, two with GA genotype, and one donor with AA genotype. All rams were kept under identical nutritional conditions and held under the same environmental conditions. Fresh semen was collected using an artificial vagina following a routine collection period of one time per week/per ram.

Human spermatozoa were obtained from voluntary male donors and data were analyzed and classified into two different groups: fertility status (fertile men, that already have progeny without resorting to medically assisted reproduction, *vs* infertile, with no progeny), and fertility treatment (spermogram, fresh sperm analysis; IIU, intra-uterine sperm injection; and ICSI, intra-cytoplasmic sperm injection). Also, the same data were analyzed and classified according to codon 174 genotype of *PRND* gene, 3'UTR genotype of the same gene and World Health Organization (WHO) criteria (World Health Organization, 2010). The classification according WHO criteria is sub-divided into asthenozoospermia (<32% progressive motility), oligozoospermia ($<15x10^6$ mL^{-1}), teratozoospermia (<4% normal sperm morphology), oligoasthenozoospermia ($<15x10^6$ m^{L-1} and <32% progressive motility), and normozoospermia (none of the above). All male donors signed a permission contained in an informed consent accordingly to their fertility treatment.

2.3.2. Evaluation of fresh semen

After semen collection, ram samples were placed in a water bath (30^{o}C) and evaluated for individual motility (progressive motile spermatozoa), concentration, vitality and morphology (Fig. 3). Evaluation of fresh semen was performed in an Olympus BX40 with an Achromatic 40x/0.65 Ph2 objective and pictures were taken with a Sony Exwave HAD DC50AP Color Video Camera with an AMCap software (v8.0). Only good quality semen (individual motility > 40%; concentration > 2,5x10^{9} mL^{-1}) was used in fresh and thawed assays (Valente *et al.*, 2010). Smear slides were performed for future characterization of ovine Doppel expression.

After collection, human semen samples were incubated for 15-60 min at room temperature for liquefaction and evaluated for individual motility (total and progressive motility), concentration, vitality and morphology (Kit Diff-Quik, Medion Diagnostics International, USA). Smear slides were also performed for future characterization of human Doppel expression.

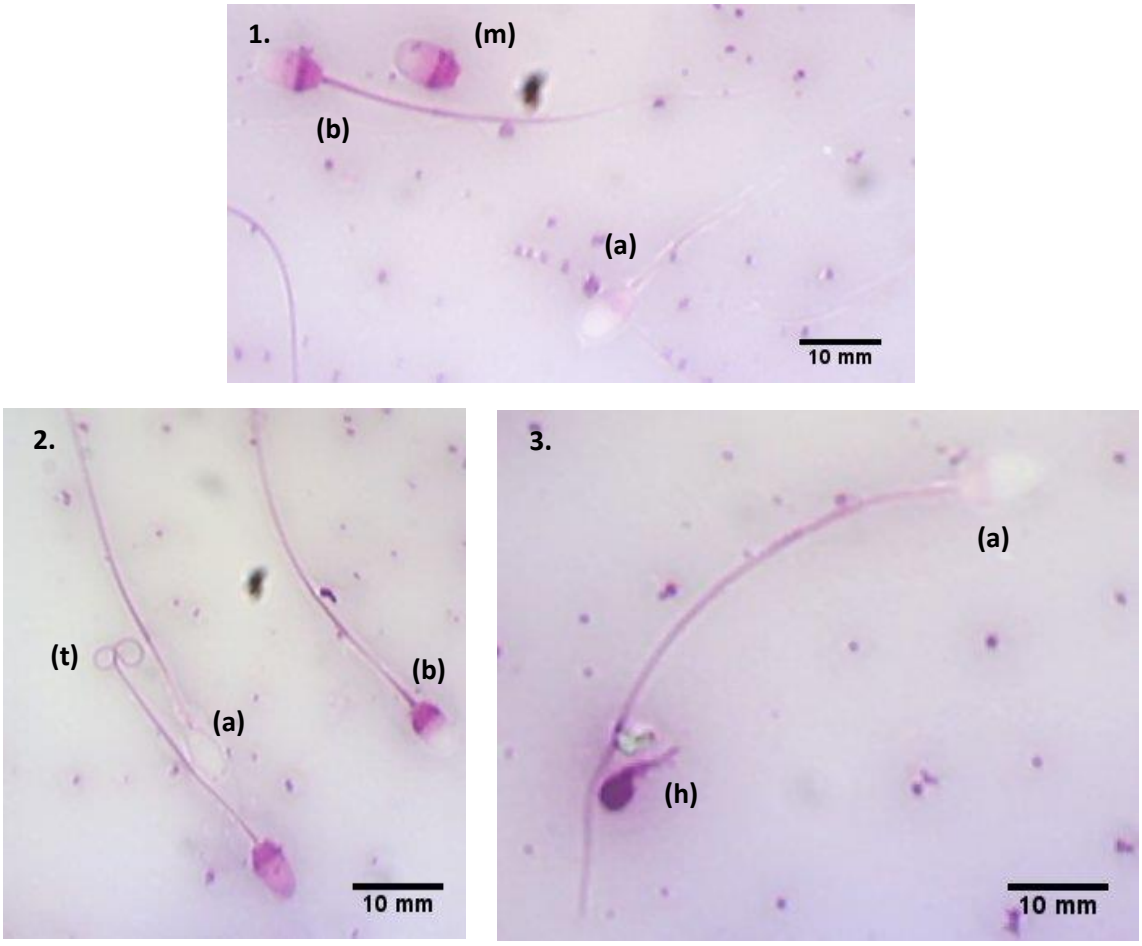

Fig. 3 – Ram spermatozoa morphology analysis with eosin-nigrosin staining.
(a) live spermatozoa; **(b**) dead spermatozoa; **(h)** head abnormality; **(t)** tail abnormality; **(m)** mid piece abnormality.

2.3.3. Semen cryopreservation

After quality assessment, ovine ejaculates were frozen in mini-straws (IMV-Technologies®, France) after dilution in cryoprotective medium (15% egg yolk and 6.5% glycerol; Table 18, Annex I) to a final concentration of 250-300x10^6 spz/per straw. The mini-straws were refrigerated for 4 h at -4^{o}C, placed in liquid nitrogen vapors for 25 min by using a Floating Rack System (Minitub®, Germany) and then submersed and stored in liquid nitrogen until future fertility assays, as in Pereira *et al.* (2009).

For the human samples, 500 µL of semen were frozen in cryotubes after 1:1 dilution in the same cryoprotective medium (Table 18, Annex I). The cryotubes were incubated for 10 min at room temperature, placed in liquid nitrogen vapor for 15 min and then submersed and kept in liquid nitrogen until quality assessment thawing were performed.

2.3.4. Evaluation of thawed semen

For the ram samples, thawing was performed by submerging the straws into a 37^{o}C water bath for 30 sec and then semen individual motility and morphology were analyzed. The thawed semen was incubated at 38.5^{o}C and 5% CO_2 for 1h in modified Bracket's medium containing 20% ovine serum for swim-up (swim-up medium; Table 19, Annex I) (Marques *et al.*, 2006). Post-swim-up sperm was centrifuged at 1500 rpm for 5 min and the supernatant rejected. The individual motility, vigor (0-5) and concentration of the remaining pellet were determined and aliquots of this semen used for capacitation status evaluation (CTC), for analysis of mitochondrial potential (JC-1) and for fertility assays.

For the human samples, cryotubes were placed at room temperature for 1-2 min for thawing process. Semen individual motility and morphology were analyzed. A motile sample of frozen-thawed sperm was obtained by centrifugation at 1200 rpm for 15 min on a density gradient: 1 mL of 50% (v/v) SupraSperm® (diluted in Universal IVF medium; Origio, Denmark) over 1 mL of 100% (v/v) SupraSperm®. The supernatant was rejected; semen pellet was resuspended in IVF medium and centrifuged for a further 5 min at 1200 rpm. Semen individual motility, concentration and vitality were analyzed in the resulting pellet. All human samples were destroyed after cryo-resistance assessment.

2.3.5. Capacitation status evaluation (CTC)

The capacitation status of ovine post-swim-up thawed semen was evaluated using chlortetracycline (CTC) staining as described by Pereira *et al.* (2009), with some modifications. Aliquots (5 µL) of this semen were placed in slides and mixed with 5 µL CTC staining solution (Table 21, Annex I), 1 µL glutaraldehyde solution (12.5%) and 1 µL 1,4-Diazabicycol (2.2.2) octane (0.22 M DABCO; Merck, Germany). Slides with coverslips were kept protected from the light and 100 cells/ejaculate were analyzed, with an Olympus BX51 using an UPlanFI 40x/0.75 NA objective and a BP 470-490 filter, within 12-24h. Spermatozoa were classified according to their acrosomal status (Fig. 4) as: F pattern (non-capacitated, with fluorescence detected over the whole region of the sperm head); B pattern (capacitated with intact acrosome, with fluorescence detected in the pre-acrosomal region of sperm head); and AR pattern (reacted acrosome, no fluorescence detected over the sperm head or an equatorial bright band). Pictures were taken in an Olympus BX60 microscope using an UPlanFL N 60x/0.9 NA objective and a BP 470-490 filter with Hamamatsu Orca R^2 monochromatic camera and using the HCI Image Live software (v3.0).

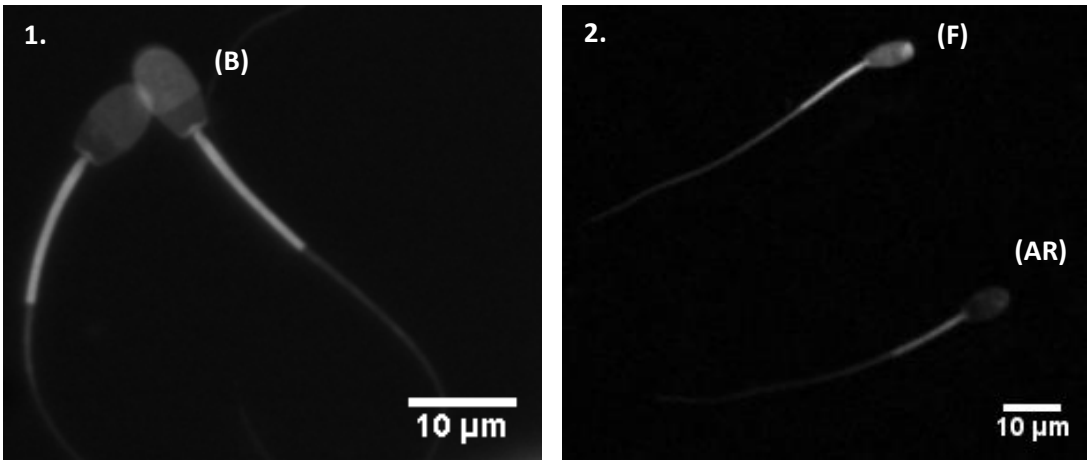

Fig. 4 – Ram spermatozoa capacitation status assessment with chlortetracyclin staining (CTC).
(B) capacitated with intact acrosome; **(F)** non-capacitated; **(AR)** acrosome reacted.

2.3.6. Mitochondrial membrane potential (JC-1)

The inner mitochondrial membrane potential of ram spermatozoa was evaluated using JC-1 staining (5,5',6,6'-tetra-chloro-1,1',3,3'-tetrathylbenzimidazolycarbocyanine iodide; Sigma-Aldrich, USA) according to a technique adapted from Gamboa *et al.* (2010). This cationic dye shows orange-red fluorescence for functional mid piece mitochondrial and green fluorescence for low inner mitochondrial membrane potential. The post-swim-up semen was diluted to $20x10^6$ mL^{-1} (Table 22) and incubated with JC-1 in dimethyl sulfoxide (2 µM DMSO; Sigma-Aldrich, USA) for 20min at 35°C. For a simultaneous vitality evaluation, 50 µL of propidium iodide (PI) were added to spermatozoa before incubation. After incubation, 5.5 µL of this semen were placed on a slide with coverslip and 100 spermatozoa were analyzed in an Olympus BX51 microscope using an UPlanFI 40x/0.75 NA objective and a BP 470-490 filter.

2.4. Fertility assays

2.4.1. Oocyte collection and In vitro fertilization

Ovine ovaries were collected from a local abattoir and were carried to the laboratory immersed in Dulbecco's phosphate buffer saline (PBS; Oxoid, Hampshire, England) supplemented with 0.15% (w/v) BSA and 0.05 mg mL^{-1} of kanamycin at 37°C. Follicles with 2-6 mm were aspirated with a 5 mL syringe and a 19G needle to obtain immature cumulus oocyte complexes (COC). Good quality COCs were selected, washed and then incubated in maturation medium (Table 24, Annex I) for 22h at 39°C in a humidified atmosphere containing 5% CO_2, as in Pereira *et al.* (2009).

Mature oocytes were washed to remove excess cumulus cells and then placed in wells containing 450 µL of IVF medium (Table 25, Annex I). Each well was inseminated with previously capacitated frozen-thawed (FT) semen at a concentration of 1×10^6 mL^{-1} and incubated for 18h at 39°C in a humidified atmosphere containing 5% CO_2 (Day 0 = Day of *in vitro* fertilization).

2.4.2. Embryo culture

After 18h of co-incubation, the presumptive zygotes were denuded in synthetic oviduct fluid (SOF; Table 23, Annex I) medium enriched with 6 mg mL^{-1} BSA, transferred to culture plates with 25 µL of zygote transfer medium (Table 26, Annex I) and incubated at 38.5°C and 5% O_2, 5% CO_2 and 90% N_2 in a humidified atmosphere until 2-4-8-cell stage (Day 2). Cleaved embryos were kept in culture medium with 0.6% BSA and 10% foetal calf serum (FCS; Sigma-Aldrich, USA; Table 27, Annex I) until the blastocyst stage (Day 6-8) without manipulation or medium exchange, as described in Pereira *et al.* (2009).

The cleavage rate was recorded on Day 2 as the number of cleavage embryos/per number of inseminated oocytes, and the embryo rate was recorded from Days 6 to 8 as the number of morulae and blastocyst/per number of cleavage embryos.

2.5. Immunolocalization

The indirect immunofluorescence was used to localize and to characterize the expression of *PRND* gene in ram and human spermatozoa. The flow cytometry was performed to assess the specificity of the two anti-Doppel monoclonal antibodies produced by mouse immunization in ram spermatozoa.

2.5.1. Indirect immunofluorescence

Fresh semen from the five rams was diluted in PBS (1:100) and 10 µL of ovine semen suspension were used to do smear slides air-dried for 10 min, fixed with methanol air-dried for 5 min and stored at -20^{o}C. Human smear slides were also prepared using 10 µL of fresh semen. This technique was adapted from Pimenta *et al.* (2012b).

The smear slides were thawed, washed three times with T-PBS and incubated in T-PBS (PBS with 0.1% (v/v) Tween-20) supplemented with 5% (w/v) BSA overnight at 4^{o}C to block nonspecific sites. Then, slides were washed three times with T-PBS with 5% BSA and incubated for 180 min at room temperature (22-24^{o}C) with two (M1 and M2) mouse anti-Doppel monoclonal antibodies (1:100 in T-PBS with 5% BSA), from two different mouse lineages, or with T-PBS with 5% BSA (used as negative controls). After this, smear slides were washed again three times with T-PBS with 5% BSA and incubated in the dark for 60 min at room temperature with FITC-conjugated secondary antibody (1:3000; Rockland Immunochemicals Inc., Pennsylvania, USA). After 30 min of incubation, 5 µL of 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Invitrogen, USA) were added to the slide for nucleic acid staining, allowing another 30 min of incubation.

Finally, slides were narrowly washed five times with T-PBS and covered with 50 µL of glycerol + T-PBS (1:1) and a coverslip. The smear slides were observed at room temperature in an Olympus BX60 microscope using a UPlanFL N 60x/0.9 NA objective with Hamamatsu Orca R2 monochromatic camera and using the HCI Image Live software (v3.0). DAPI fluorescence was detected using a BP 330-385 excitation filter and FITC with a BP 470-490 excitation filter.

2.5.2. Flow cytometry

Aliquots of fresh and FT semen were washed and centrifuged twice with PBS supplemented with 1% BSA (Sigma-Aldrich, USA) at 1500 rpm for 10 min at room temperature. Supernatant was rejected and 100 µL of diluted semen (50 000 mL^{-1}) in PBS with 1% BSA were incubated with two different anti-Doppel monoclonal primary antibodies (1:100) previously produced (M1 and M2), or with PBS with 1% BSA (used as negative control) for 180 min at 4°C. Then, spermatozoa were washed and centrifuged twice with PBS at 1500 rpm for 10 min. Supernatant was rejected and 40 µL of FITC-conjugated secondary antibody (1:100; Rockland Immunochemicals Inc., Pennsylvania, USA) were added and incubated for 45 min at 4°C.

Sperm suspensions were diluted at $1x10^6$ mL^{-1} and fluorescence was measured on a FacsCalibur flow cytometer (Becton-Dickinson Biosciences, USA) equipped with a 488 nm argon-ion laser. Measurements for at least 20 000 events were collected per sample. Data were analyzed using CellQuest software (Becton-Dickinson Biosciences, USA).

This technique was used to distinguish ovine Doppel on fresh and FT spermatozoa from rams of the three previously described *PRND* genotypes, but also to select the most specific anti-Doppel monoclonal antibody.

2.6. Statistical analysis

The results were expressed as least square means ± standard error. The genotype frequencies of codon 174 and 3'UTR of human *PRND* gene were calculated and possible deviations from Hardy-Weinberg equilibrium were evaluated using Genepop software (v3.4; Laboratoire de Genetique et Environment, Montpellier, France).

Data representing 3-17 replicates of spermatozoa quality and morphological parameters, mitochondrial membrane potential and capacitation status, as well as 4 replicates for IVF assays, were analyzed using the MIXED procedure of Statistical Analysis Systems Institute (SAS Inst., Inc., Cary, NC, USA). The mixed linear model included *PRND* genotypes as fixed effect and replicates as random effect. Data from flow cytometry measurements were analyzed using the same procedure of SAS but considering also the semen status (fresh or frozen-thawed) as fixed effect. Human data of spermatozoa quality and morphological parameters were equally analyzed including *PRND* genotypes or fertility status or fertility treatments as fixed effect and replicates as random effect. When significant effects were identified, values were compared using the PDIFF test. Differences were considered significant when $P \leq 0.05$.

3. RESULTS

3.1. *PRND* polymorphisms analysis

3.1.1. MRF-SSCP

Three different patterns were identified for exon 2 of ovine *PRND* gene (Fig. 5) by MRF-SSCP, which match the patterns previously described by Pereira *et al.* (2009). This identification allowed the selection of 5 rams based on their *PRND* genotype (2 with GG genotype, 2 with GA genotype, and 1 with AA genotype) to be used in the fertility assays.

In human samples, four different patterns (A-D) in exon 2 of human *PRND* gene (Fig. 6) were observed, also by MRF-SSCP. However, in spite of trying several MRF-SSCP conditions, it was impossible to obtain really discriminating patterns. Thus, human samples were sequenced for polymorphism confirmation.

Fig. 5 – Multiple restriction fragment - single-strand conformation polymorphism of ovine *PRND* gene. Three MRF-SSCP patterns were identified in this study: GG, GA and AA.

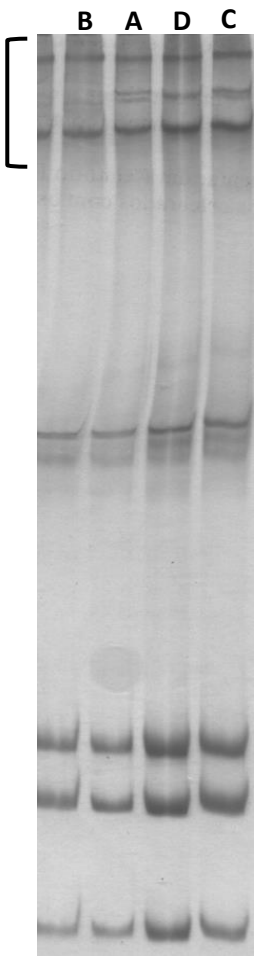

Fig. 6 – Multiple restriction fragment - single-strand conformation polymorphism of human *PRND* gene. Four MRF-SSCP patterns were observed in this study: A, B, C and D.

3.1.2. Sequencing

Two polymorphisms were detected in the human *PRND* gene (Fig. 7a – d), within the coding sequence of exon 2. A C→T mutation at the second position of codon 174 is responsible for an amino acid exchange from threonine to methionine (T174M), which matches the mutation described in Peoc'h *et al.* (2000) and *Schröder et al.* (2001) (Fig. 7a – c). Also, a rare polymorphism was observed in 1/28 cases and a G→A non-coding change at the third position of codon 174 was also detected (Fig. 7d; Mead *et al.*, 2000). Another polymorphism outside the coding sequence was also identified (Fig. 8).

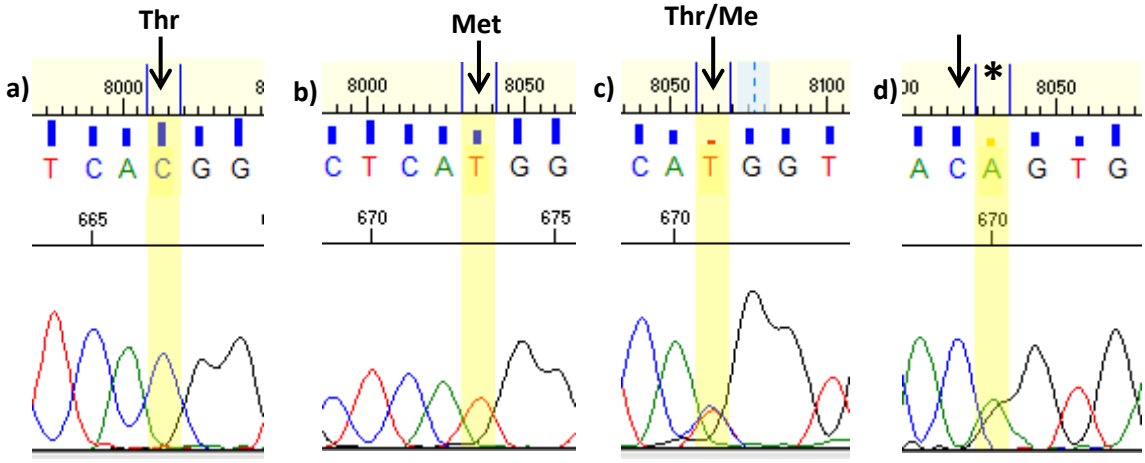

Fig. 7 – Electropherograms of human *PRND* codon 174 polymorphism.
The nucleotide exchange at the second position is indicated by arrow; the nucleotide exchange at the third position is indicated by asterisk. **a)** Wild-type sequence (CC) - threonine; **b)** mutant genotype (TT) - methionine; **c)** heterozygous genotype (CT); **d)** non-coding change at the third position of codon 174.

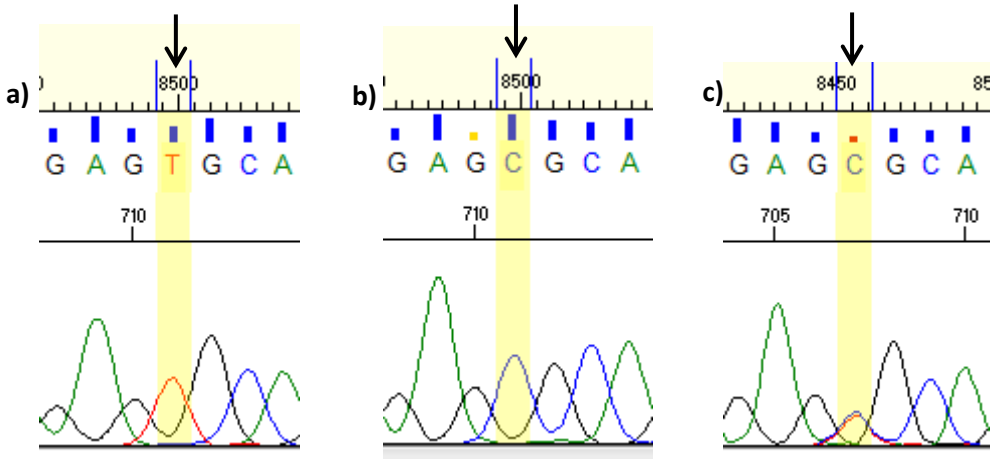

Fig. 8 – Electropherograms of human *PRND* 3' UTR polymorphism.
The nucleotide exchange is indicated by arrows. **a)** Wild-type sequence (TT); **b)** mutant genotype (CC); **c)** heterozygous genotype (TC).

The genotype distribution of both codon 174 and 3' UTR polymorphisms of *PRND* gene (Table 3 and 4, respectively) was very similar among the male donors. Therefore, there were no differences (P>0.05) in genotype distribution between fertile status nor among different treatments (sperm analysis, ICSI or IIU) for both codon 174 (Table 3) and 3' UTR polymorphisms (Table 4). However, it was possible to notice an association between the two polymorphisms, indicating they are in close association (P=0.018; Table 5). This association was also described by Mead *et al.*, (2000). The *PRND* T allele of 3'UTR polymorphism is significantly linked to the *PRND* C allele of codon 174 (P≤0.0001; Table 5), whereas the *PRND* C allele of 3'UTR polymorphism is significantly associated with *PRND* T allele of codon 174 (P=0.001; Table 5).

Table 3 - Genotype distribution at codon 174 of human *PRND* gene.

		Codon 174 genotypes			Total of individuals
		CC	CT	TT	
Fertility status	**Fertile**	1 (33%)	2 (67%)	0 (0%)	3/29
	Infertile	6 (23%)	11 (42%)	9 (35%)	26/29
Fertility treatment	**Spermogram**	3 (18%)	10 (59%)	4 (24%)	17/29
	ICSI	1 (20%)	1 (20%)	3 (60%)	5/29
	IIU	3 (43%)	2 (29%)	2 (29%)	7/29
WHO criteria	**Norm**	5 (29%)	9 (53%)	3 (18%)	17/29
	AZ	1 (33%)	0 (0%)	2 (67%)	3/29
	OZ	1 (25%)	1 (25%)	2 (50%)	4/29
	TZ	0 (0%)	2 (67%)	1 (33%)	3/29
	OAZ	0 (0%)	1 (50%)	1 (50%)	2/29

Codon 174 genotypes: **CC**, wild-type genotype; **CT**, heterozygous genotype; **TT**, homozygous mutant genotype. **Fertile**, male donors that already have progeny without resorting to medically assisted reproduction; **Infertile**, male donors with no progeny; **Spermogram**, fresh sperm analysis; **ICSI**, intra-cytoplasmic sperm injection: **IIU**, intra-uterine sperm injection. WHO criteria: **AZ**, asthenozoospermia; **OZ**, oligozoospermia; **TZ**, teratozoospermia; **OAZ**, oligoasthenozoospermia; **Norm**, normozoospermia.

Table 4 - Genotype distribution of 3'UTR polymorphism of human *PRND* gene.

		3' UTR polymorphism genotypes			Total of individuals
		TT	TC	CC	
Fertility status	**Fertile**	1 (33%)	2 (67%)	0 (0%)	3/29
	Infertile	6 (23%)	10 (38%)	10 (38%)	26/29
Fertility treatment	**Spermogram**	3 (18%)	9 (53%)	5 (29%)	17/29
	ICSI	1 (20%)	1 (20%)	3 (60%)	5/29
	IIU	3 (43%)	2 (29%)	2 (29%)	7/29
WHO criteria	**Norm**	5 (29%)	9 (53%)	3 (18%)	17/29
	AZ	1 (33%)	0 (0%)	2 (67%)	3/29
	OZ	1 (25%)	1 (25%)	2 (50%)	4/29
	TZ	0 (0%)	2 (67%)	1 (33%)	3/29
	OAZ	0 (0%)	0 (0%)	2 (100%)	2/29

3' UTR polymorphism genotypes: **TT**, wild-type genotype; **TC**, heterozygous genotype; **CC**, homozygous mutant genotype. **Fertile**, male donors that already have progeny without resorting to medically assisted reproduction; **Infertile**, male donors with no progeny; **Spermogram**, fresh sperm analysis; **ICSI**, intra-cytoplasmic sperm injection: **IIU**, intra-uterine sperm injection. WHO criteria: **AZ**, asthenozoospermia; **OZ**, oligozoospermia; **TZ**, teratozoospermia; **OAZ**, oligoasthenozoospermia; **Norm**, normozoospermia.

Table 5 – Genotype frequencies relative to single nucleotide polymorphisms detected in codon 174 and 3' UTR polymorphisms of the human *PRND* gene, in 29 Portuguese male donors.

		3' UTR genotypes		
		TT	TC	CC
Codon 174 genotypes	**CC**	1.000 a	0.000 a	0.000 a
	CT	0.000 b	0.923 b	0.077 b
	TT	0.000 c	0.000 c	1.000 c

3' UTR genotypes: **TT**, wild-type genotype; **TC**, heterozygous genotype; **CC**, homozygous mutant genotype.
Codon 174 genotypes: **CC**, wild-type genotype; **CT**, heterozygous genotype; **TT**, homozygous mutant genotype. Values within columns with different letters differ statistically ($P<0.001$).

3.2. Fertility tests

The volume, individual motility (MI) and vitality of ovine fresh semen presented no differences (P>0.05) among animals with different *PRND* genotypes (Table 6). However, animals with *PRND* AA genotype showed lower sperm concentration in fresh ejaculates when compared to animals with *PRND* GA (P<0.006) and GG (P<0.008) genotypes (Table 6).

Table 6 – Quality parameters evaluation of fresh semen from Churra Galega Mirandesa rams classified according to codon 26 genotype of *PRND* gene (least squares means ± standard error).

PRND genotype	n	Volume (mL)	Concent (10^9 mL^{-1})	MI (%)	Vitality (%)
AA	7	0.4 ± 0.10	2.4 ± 0.50 a	63.3 ± 2.59	75.3 ± 6.49
GA	17	0.7 ± 0.06	4.3 ± 0.30 b	63.6 ± 1.73	68.5 ± 3.88
GG	11	0.5 ± 0.08	4.3 ± 0.36 b	61.0 ± 1.91	68.5 ± 4.81

AA, rare homozygous mutant; **GA**, heterozygous mutant; **GG**, homozygous wild-type; **n**, number of samples; **Concent**, spermatozoa concentration; **MI,** individual spermatozoa motility; Values within columns with different letters differ statistically (P≤0.008)

The thawed and post-swim-up MI, vigor, concentration, vitality and inner mitochondrial membrane potential from post-swim-up frozen-thawed (FT) ram semen (Table 7), as well as the capacitation status (Table 8), presented no differences (P>0.05) among rams with distinct *PRND* genotypes.

Table 7 – Quality parameters evaluation of frozen-thawed semen from Churra Galega Mirandesa rams classified according to codon 26 genotype of *PRND* gene (least squares means ± standard error).

PRND genotype	n	Thawed	Post-swim-up					
		MI (%)	MI (%)	Vigor (0-5)	Concent ($x10^6$ mL^{-1})	Vitality (%)	n	Δψmit (%)
AA	7	44.0 ± 3.22	66.9 ± 6.24	4.2 ± 0.19	66.9 ± 57.55	38.2 ± 5.38	4	54.3 ± 10.41
GA	21	41.5 ± 1.92	53.3 ± 3.94	4.2 ± 0.13	126.2 ± 34.89	51.2 ± 3.47	13	50.4 ± 5.66
GG	19	40.2 ± 2.01	53.4 ± 4.02	4.3 ± 0.13	197.8 ± 35.69	43.8 ± 4.02	13	66.9 ± 5.63

AA, rare homozygous mutant; **GA**, heterozygous mutant; **GG**, homozygous wild-type; **n**, number of samples; **MI**, individual spermatozoa motility; **Concent**, spermatozoa concentration; **Δψmit**, inner mitochondrial membrane potential.

Table 8 – Capacitation status evaluation of frozen-thawed semen from Churra Galega Mirandesa rams classified according to codon 26 genotype of *PRND* gene (least squares means ± standard error).

PRND genotype	n	Capacitation status (%)		
		F	B	AR
AA	7	26.2 ± 4.32	60.5 ± 7.43	17.2 ± 6.85
GA	21	18.6 ± 2.90	57.4 ± 6.62	22.8 ± 5.86
GG	19	19.4 ± 2.98	57.5 ± 6.66	22.5 ± 5.90

AA, rare homozygous mutant; **GA**, heterozygous mutant; **GG**, homozygous wild-type; n, number of samples; **F**, non-capacitated; **B**, capacitated with intact acrosome; **AR**, reacted acrosome.

Semen morphological evaluation showed that rams with *PRND* GG genotype had more (P=0.04) morphological abnormalities in fresh samples when compared to rams with GA *PRND* genotype (Table 9). Also, spermatozoa mid piece abnormalities were higher in fresh semen from animals with *PRND* GG genotype when compared to samples from rams carrying *PRND* AA (P=0.02) and GA (P=0.001) genotype (Table 9). In FT samples, there were no significant differences (P>0.05) between animals with distinct *PRND* genotypes.

Table 9 – Morphological evaluation of fresh (F) and frozen-thawed (FT) semen from Churra Galega Mirandesa rams classified according to codon 26 genotype of *PRND* gene (least squares means ± standard error).

PRND genotype		n	Sperm morphology (%)			
			Normal	Head	Mid piece	Tail
Fresh	**AA**	6	84.7 ± 3.09 ab	4.8 ± 2.34	2.7 ± 1.16 a	6.2 ± 2.30
	GA	17	88.7 ± 2.06 a	6.7 ± 1.53	3.1 ± 0.69 a	1.9 ± 1.53
	GG	11	83.0 ± 2.18 b	7.6 ± 1.76	6.7 ± 0.86 b	3.5 ± 1.62
FT	**AA**	6	79.2 ± 4.68	13.2 ± 3.79	2.4 ± 1.78	5.2 ± 1.91
	GA	13	77.1 ± 3.09	16.4 ± 2.48	2.5 ± 1.13	4.4 ± 1.26
	GG	11	73.8 ± 3.24	16.2 ± 2.62	6.0 ± 1.22	4.0 ± 1.32

AA, rare homozygous mutant; **GA**, heterozygous mutant; **GG**, homozygous wild-type; **n**, number of samples; **Normal**, normal spermatozoa morphology; **Head**, spermatozoa head defect; **Mid piece**, spermatozoa mid piece defect; **Tail**, spermatozoa tail defect; **Fresh**, fresh semen; **FT**, frozen-thawed semen; Values within columns with different letters differ statistically (P≤0.02)

No differences were observed in cleavage rate (P>0.05) after fertilization with FT semen from rams with distinct *PRND* genotypes (Table 10; Fig. 9). However, rams carrying *PRND* AA genotype showed higher (P<0.004) embryo rate at day 6/7 when compared to rams with *PRND* GG or GA genotype (Table 10).

Table 10 – IVF parameters evaluation of fertilization with frozen-thawed semen from Churra Galega Mirandesa rams classified according to codon 26 genotype of *PRND* gene (least squares means ± standard error).

PRND genotype	n	Cleavage rate (%)	D6/7 embryo rate (%)
AA	372	40.4 ± 3.69	26.6 ± 3.32 a
GA	909	37.0 ± 2.99	14.7 ± 2.39 b
GG	861	40.2 ± 3.00	15.2 ± 2.31 b

AA, rare homozygous mutant; **GA**, heterozygous mutant; **GG**, homozygous wild-type; **n**, number of inseminated oocytes; Values within columns with different letters differ statistically (P≤0.004)

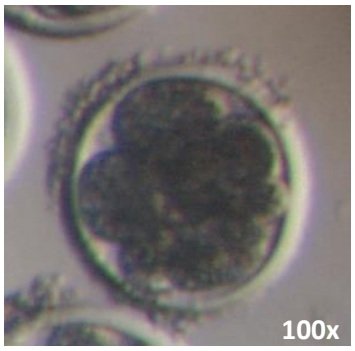

Fig. 9 – Ovine cleaved embryo (day 2)

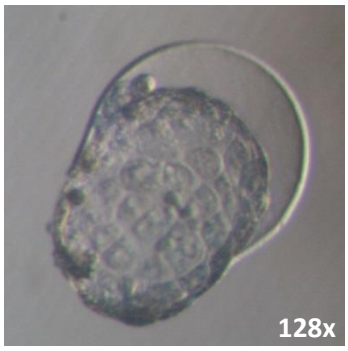

Fig. 10 – Ovine hatching blastocyst (day 6/7)

In what concerns human results, quality evaluation of fresh sperm only highlighted that fertile male donors had less (P=0.03) sperm volume, comparing to infertile ones (Table 11). Also, fresh sperm concentration tend to be superior (P=0.08) in fertile male donors than in infertile ones (Table 11). Moreover, total MI, progressive MI and vitality presented no differences (P>0.05) among donors classified according to their distinct sperm analysis (fertility status, fertility treatment) or *PRND* genotypes (codon 174 and 3'UTR polymorphisms; Table 11).

Frozen-thawed sperm evaluation presented no differences (P>0.05) among different sperm analysis (Table 12). However, FT sperm vitality tends to be superior (P=0.09) in samples from male donors carrying CC genotype of codon 174 polymorphism (Table 12).

Table 11 – Quality parameters evaluations of fresh semen from Portuguese human male donors classified according to their fertility status, fertility treatment, codon 174 or 3'UTR genotypes of *PRND* (least squares means ± standard error).

		n	Volume (mL)	Concent (10^6 mL^{-1})	Total MI (%)	Progressive MI (%)	Vitality (%)
Fertility status	**Fertile**	3	1.3 ± 0.60 a	93.5 ± 24.09	48.3 ± 6.61	40.5 ± 6.61	75.7 ± 6.71
	Infertile	26	2.8 ± 0.12 b	46.5 ± 8.18	47.3 ± 2.25	38.4 ± 2.33	73.1 ± 2.28
Fertility treatment	**Spermogram**	17	2.6 ± 0.31	56.2 ± 10.85	46.4 ± 2.81	37.1 ± 2.90	74.8 ± 2.85
	ICSI	5	1.8 ± 0.51	43.6 ± 20.00	53.2 ± 5.10	43.3 ± 5.12	70.8 ± 5.25
	IIU	7	2.9 ± 0.44	45.3 ± 16.90	45.3 ± 4.32	38.6 ± 4.36	71.9 ± 4.44
Codon 174 genotypes	**CC**	7	2.0 ± 0.43	46.3 ± 16.73	50.7 ± 4.31	37.8 ± 4.28	81.0 ± 4.14
	CT	13	2.9 ± 0.33	60.7 ± 12.35	47.7 ± 3.16	40.6 ± 3.20	71.8 ± 3.03
	TT	9	2.6 ± 0.41	41.7 ± 14.03	44.4 ± 3.80	36.1 ± 3.91	69.8 ± 3.65
3'UTR genotypes	**TT**	7	2.0 ± 0.43	46.2 ± 16.83	50.7 ± 4.25	37.8 ± 4.23	81.0 ± 4.14
	TC	12	2.9 ± 0.34	59.7 ± 12.88	48.8 ± 3.24	41.5 ± 3.31	71.7 ± 3.16
	CC	10	2.6 ± 0.40	44.9 ± 14.17	43.5 ± 3.55	35.3 ± 3.78	70.1 ± 3.46

Fertile, male donors that already have progeny without resorting to medically assisted reproduction; **Infertile**, male donors with no progeny; **Spermogram**, fresh sperm analysis; **ICSI**, intra-cytoplasmic sperm injection: **IIU**, intra-uterine sperm injection; Codon 174 genotypes: **CC**, wild-type genotype; **CT**, heterozygous genotype; **TT**, homozygous mutant genotype; 3' UTR genotypes: **TT**, wild-type genotype; **TC**, heterozygous genotype; **CC**, homozygous mutant genotype; **n**, number of samples from male donors; **Concent**, spermatozoa concentration; **Total MI,** spermatozoa individual motility, including progressive motility; **Progressive MI**, spermatozoa with straight line motility; Values within columns with different letters differ statistically (P<0.03)

Table 12 – Quality parameters evaluations of frozen-thawed semen from Portuguese human male donors classified according to their fertility status, fertility treatment, codon 174 or 3'UTR genotypes of *PRND* (least squares means ± standard error).

		n	Thawed	After centrifugation with density gradient		
			MI (%)	MI (%)	Concent (10^6 mL^{-1})	Vitality (%)
Fertility status	Fertile	3	27.3 ± 7.08	46.5 ± 14.56	6.3 ± 5.04	34.5 ± 13.07
	Infertile	26	15.6 ± 2.76	36.1 ± 6.24	7.0 ± 2.09	29.4 ± 5.40
Fertility treatment	Spermogram	17	19.6 ± 2.94	36.8 ± 6.59	7.4 ± 2.31	30.5 ± 5.77
	ICSI	5	9.3 ± 5.88	32.6 ± 11.08	5.3 ± 4.04	21.2 ± 10.38
	IIU	7	14.3 ± 4.45	41.9 ± 8.83	6.4 ± 3.17	32.8 ± 8.06
Codon 174 genotype	CC	7	17.5 ± 4.91	45.6 ± 8.66	6.4 ± 3.22	42.0 ± 7.63
	CT	13	19.3 ± 3.40	36.8 ± 6.53	7.9 ± 2.42	30.2 ± 5.62
	TT	9	12.5 ± 4.40	30.4 ± 8.32	5.3 ± 3.09	18.5 ± 7.24
3'UTR genotype	TT	7	17.5 ± 4.78	45.7 ± 8.46	6.3 ± 3.18	41.9 ± 7.79
	TC	12	20.4 ± 3.41	38.7 ± 6.45	5.9 ± 2.41	29.1 ± 5.87
	CC	10	11.4 ± 4.01	26.8 ± 7.96	9.3 ± 2.97	20.8 ± 7.25

Fertile, male donors that already have progeny without resorting to medically assisted reproduction; **Infertile**, male donors with no progeny; **Spermogram**, fresh sperm analysis; **ICSI**, intra-cytoplasmic sperm injection: **IIU**, intra-uterine sperm injection; Codon 174 genotypes: **CC**, wild-type genotype; **CT**, heterozygous genotype; **TT**, homozygous mutant genotype; 3' UTR genotypes: **TT**, wild-type genotype; **TC**, heterozygous genotype; **CC**, homozygous mutant genotype; **n**, number of samples from male donors; **Concent**, spermatozoa concentration; **MI,** individual spermatozoa motility.

No differences (P>0.05) were found in fresh (Table 13) or FT (Table 14) human sperm morphological analysis. Still, FT samples from fertile male donors tend to possess more (P=0.07) sperm mid piece abnormalities (Table 14).

Table 13 – Morphological evaluation of fresh semen from human male donors classified according to their fertility status, fertility treatment, codon 174 or 3'UTR genotypes of *PRND* (least squares means ± standard error)

		n	Fresh sperm morphology (%)			
			Normal	Head	Mid piece	Tail
Fertility status	Fertile	3	12.9 ± 5.02	64.1 ± 13.66	11.8 ± 6.12	10.4 ± 9.17
	Infertile	26	8.1 ± 2.21	74.7 ± 6.14	8.1 ± 2.76	9.6 ± 3.97
Fertility treatment	Spermogram	17	7.9 ± 1.95	76.0 ± 6.47	7.0 ± 2.78	9.8 ± 4.40
	ICSI	5	11.2 ± 2.33	74.7 ± 8.61	7.9 ± 3.68	6.4 ± 6.43
	IIU	7	8.6 ± 2.19	65.2 ± 7.76	13.0 ± 3.33	12.0 ± 5.63
Codon 174 genotype	CC	7	9.3 ± 2.13	72.9 ± 8.00	11.4 ± 3.47	5.8 ± 5.73
	CT	13	7.8 ± 1.97	75.7 ± 6.86	6.9 ± 2.98	10.2 ± 4.66
	TT	9	9.1 ± 2.16	68.2 ± 8.07	8.9 ± 3.50	13.0 ± 5.65
3'UTR genotype	TT	7	9.3 ± 2.13	72.9 ± 8.08	11.4 ± 3.51	5.8 ± 5.74
	TC	12	8.0 ± 1.99	73.8 ± 7.07	8.1 ± 3.12	10.6 ± 4.84
	CC	10	8.8 ± 2.18	71.4 ± 8.23	6.7 ± 3.60	12.2 ± 5.66

Fertile, male donors that already have progeny without resorting to medically assisted reproduction; **Infertile**, male donors with no progeny; **Spermogram**, fresh sperm analysis; **ICSI**, intra-cytoplasmic sperm injection: **IIU**, intra-uterine sperm injection; Codon 174 genotypes: **CC**, wild-type genotype; **CT**, heterozygous genotype; **TT**, homozygous mutant genotype; 3' UTR genotypes: **TT**, wild-type genotype; **TC**, heterozygous genotype; **CC**, homozygous mutant genotype; **n**, number of samples from male donors; **Normal**, normal spermatozoa morphology; **Head**, spermatozoa head defect; **Mid piece**, spermatozoa mid piece defect; **Tail**, spermatozoa tail defect.

Table 14 – Morphological evaluation of frozen-thawed semen from human male donors classified according to their fertility status, fertility treatment, codon 174 or 3'UTR genotypes of *PRND* (least squares means ± standard error)

		n	Frozen-thawed sperm morphology (%)			
			Normal	Head	Mid piece	Tail
Fertility status	Fertile	3	10.3 ± 3.63	63.7 ± 8.74	17.3 ± 3.00	8.7 ± 5.53
	Infertile	26	7.8 ± 1.28	69.4 ± 3.17	11.3 ± 1.06	11.0 ± 1.95
Fertility treatment	Spermogram	17	8.8 ± 1.54	66.8 ± 3.77	12.6 ± 1.37	10.8 ± 2.44
	ICSI	5	10.8 ± 3.08	67.3 ± 7.54	13.3 ± 2.75	8.8 ± 4.88
	IIU	7	5.1 ± 2.33	73.3 ± 5.70	9.7 ± 2.08	11.9 ± 3.69
Codon 174 genotype	CC	7	10.5 ± 2.49	67.5 ± 6.28	12.2 ± 2.27	9.8 ± 3.97
	CT	13	8.8 ± 1.69	68.9 ± 4.28	10.9 ± 1.54	10.0 ± 2.70
	TT	9	5.1 ± 2.16	69.0 ± 5.48	13.4 ± 1.97	12.6 ± 3.44
3'UTR genotype	TT	7	10.5 ± 2.45	67.5 ± 6.25	12.2 ± 2.31	9.8 ± 3.89
	TC	12	9.3 ± 1.74	70.2 ± 4.43	11.7 ± 1.64	8.9 ± 2.75
	CC	10	5.0 ± 2.00	67.2 ± 5.14	12.1 ± 1.89	13.8 ± 3.18

Fertile, male donors that already have progeny without resorting to medically assisted reproduction; **Infertile**, male donors with no progeny; **Spermogram**, fresh sperm analysis; **ICSI**, intra-cytoplasmic sperm injection: **IIU**, intra-uterine sperm injection; Codon 174 genotypes: **CC**, wild-type genotype; **CT**, heterozygous genotype; **TT**, homozygous mutant genotype; 3' UTR genotypes: **TT**, wild-type genotype; **TC**, heterozygous genotype; **CC**, homozygous mutant genotype; **n**, number of samples from male donors; **Normal**, normal spermatozoa morphology; **Head**, spermatozoa head defect; **Mid piece**, spermatozoa mid piece defect; **Tail**, spermatozoa tail defect.

The association between human sperm traits and the linkage of codon 174 and 3'UTR polymorphisms were analyzed and no differences (P>0.05) were established (data not shown). Nevertheless, the absence of linkage between the two polymorphisms exhibited higher (P<0.001; Table 15) FT viable sperm concentration after centrifugation with density gradient, when compared to the existence of the same linkage (Table 15).

Table 15 – Relationship between human FT sperm concentration and the linkage of codon 174 and 3'UTR polymorphisms or human *PRND* gene (least squares means ± standard error)

Linkage between polymorphisms		n	Concent (10^6 mL^{-1})
Codon 174 genotypes	3'UTR genotypes		
CC	TT	7	4.9 ± 2.31 a
CT	TC	12	6.1 ± 2.02 a
TT	CC	9	6.8 ± 2.39 a
CT	TT	1	31.6 ± 4.89 b

Codon 174 genotypes: **CC**, wild-type genotype; **CT**, heterozygous genotype; **TT**, homozygous mutant genotype; 3' UTR genotypes: **TT**, wild-type genotype; **TC**, heterozygous genotype; **CC**, homozygous mutant genotype; **Concent**, spermatozoa concentration; Values within columns with different letters differ statistically (P<0.001)

3.3. Indirect immunofluorescence

Ovine Doppel protein was detected in ejaculated ram spermatozoa. Identical patterns were observed in ovine Doppel immunofluorescence localization using M1 or M2 antibodies. Positive fluorescence in sperm head apical region, mid piece and tail were observed (Fig. 11a and b). However, occasionally a faint immunofluorescence staining was also present on the flagella of ram spermatozoa in the negative control (Fig. 11c). Also, sometimes the fluorescence in sperm head apical region showed a faded positivity (Fig. 11b), so it was difficult to detect differences among samples from rams classified according to their *PRND* genotypes.

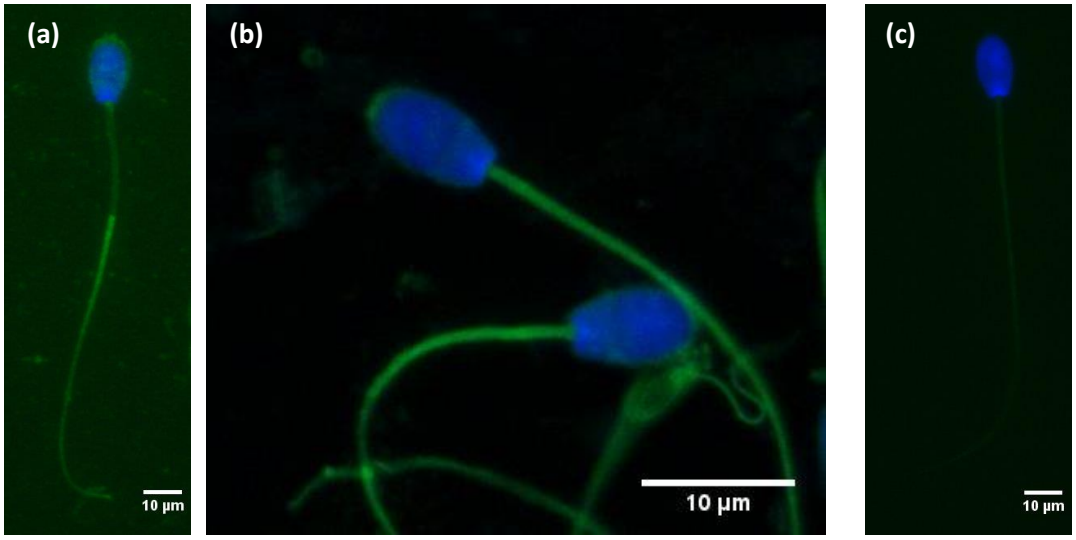

Fig. 11 – Immunofluorescence images of Doppel protein location in ram ejaculated spermatozoa.
(a) and **(b)** M1 in spermatozoa from rams with different *PRND* genotype **(c)** T-PBS, used as negative control. FITC-conjugated secondary antibody (green), DAPI (nucleus/nucleus), scale bar: 10 µm.

Identical patterns between M1 and M2 antibodies were observed in human Doppel immunofluorescence localization. Shifting positive fluorescence in sperm head and mid piece was detected in human spermatozoa (Fig. 12a and c), and a faded positivity in human sperm flagella was also observed (Fig. 12c). Nonetheless, this fluorescence patterns were also observed in the negative controls (Fig. 12b and d). Due to the similar fluorescence signal among positive and negative human samples, it was difficult to correlate the fluorescence patterns with human Doppel expression.

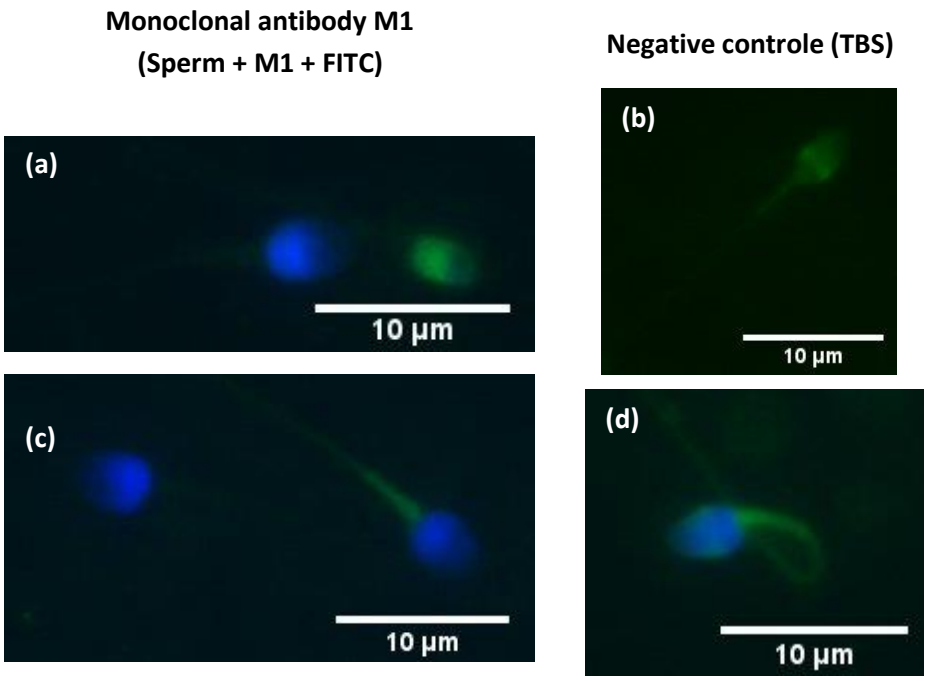

Fig. 12 - Immunofluorescence images of Doppel protein location in human ejaculated spermatozoa.
(a) and **(c)** T-PBS, used as negative control; **(b)** and **(d)** M1 in spermatozoa from human male donors with different *PRND* polymorphisms. FITC-conjugated secondary antibody (green), DAPI (nucleus/blue), scale bar: 10 µm.

3.4. Flow cytometry

Ovine Doppel protein was detected in ram spermatozoa by flow cytometry through an increase in the fluorescent signal using both produced antibodies M1 and M2 (Fig. 13 and 14). As control, we used the fluorescent signal obtained with ram spermatozoa + FITC-conjugated secondary antibody. As described in Table 16, the M1 monoclonal antibody exhibited superior fluorescent signal when compared to M2.

No differences (P>0.05) in ovine Doppel detection were identified among sperm samples from animals with different *PRND* genotypes (Table 16). However, these results also showed the detection of a superior Doppel relative fluorescence when analyzing ram fresh semen (Fig. 14a), compared to FT semen samples (Fig. 14b), using both M1 and M2 (P<0.03 and P<0.05, respectively; Table 16).

Table 16 – Fluorescent signals obtained with anti-Doppel monoclonal antibodies M1 and M2, for ovine fresh and FT semen, and for semen of Churra Galega Mirandesa rams classified according to codon 26 genotype of *PRND* gene (least squares means ± standard error).

Anti-Doppel monoclonal antibody	n	*PRND* genotypes			Semen	
		AA	GA	GG	Fresh	FT
M1	14	1.63 ± 0.30	1.40 ± 0.18	1.59 ± 0.20	1.87 ± 0.17 a	1.21 ± 0.19 b
M2	14	1.44 ± 0.43	1.18 ± 0.29	1.60 ± 0.29	1.70 ± 0.22 a	1.12 ± 0.24 b

M1, anti-Doppel monoclonal antibody M1-2C10; **M2**, anti-Doppel monoclonal antibody M2-15.5; **AA**, rare homozygous mutant; **GA**, heterozygous mutant; **GG**, homozygous wild-type; **n**, number of samples; **Fresh**, fresh semen; **FT**, frozen-thawed semen; Values within rows with different letters differ statistically (P≤0.05)

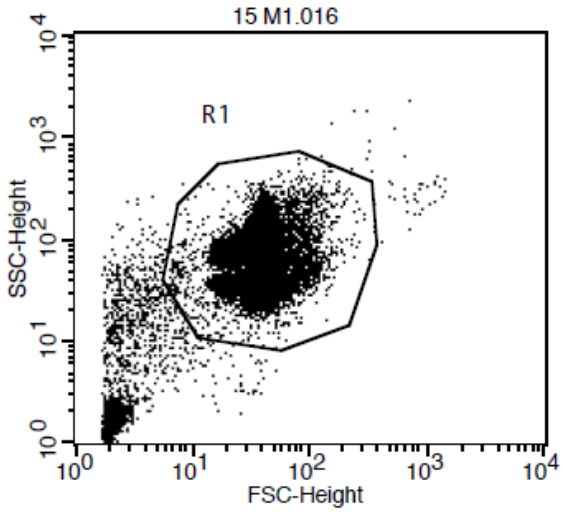

Fig. 13 – Flow cytometry of correctly oriented fresh sperm (region R1) from ram with *PRND* AA genotype (forward scatter versus side scatter).

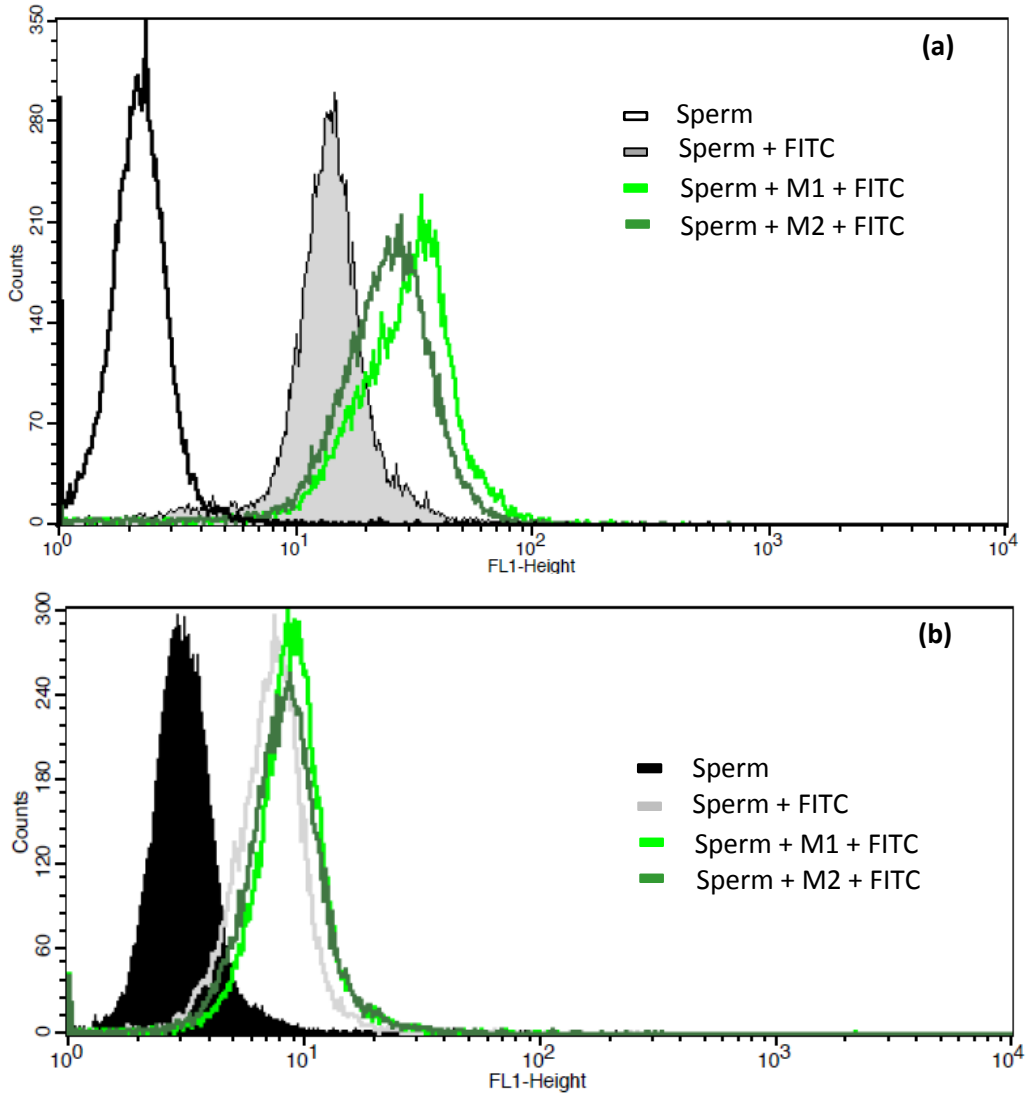

Fig. 14 – Flow cytometry of fresh and frozen-thawed sperm from ram with *PRND* AA genotype.
(a) R1 histogram output for fresh spermatozoa; **(b)** R1 output for FT spermatozoa. Fluorescent signals were detected for spermatozoa (Sperm), spermatozoa plus FITC-conjugated secondary antibody (Sperm + FITC), spermatozoa plus M1 antibody (Sperm + M1 + FITC) and spermatozoa plus M2 antibody (Sperm + M2 + FITC).

4. DISCUSSION

The prion genetic complex comprises one prion gene and three prion-like genes which show similar genetic organization, although their biological functions remain largely unclear (Premzl and Gamulin, 2007; Pimenta *et al.*, 2011). Since the surprising discovery of *PRND*, located downstream *PRNP* (Moore *et al.*, 1999), several authors have suggested a major role for Doppel in the reproductive field. Doppel protein can be found in Sertoli and germ cells in several different species, including ovine (Espenes *et al.*, 2005) and human (Peoc'h *et al.*, 2002), in Leydig cells (Kocer *et al.*, 2007) and also in epididymis epithelial cells (Serres *et al.*, 2006). All these results, combined with the sterility presented by *PRND*$^{0/0}$ mice (Behrens *et al.*, 2002), point to an important physiological function on male fertility (Peoc'h *et al.*, 2002; Rondena *et al.*, 2005; Serres *et al.*, 2006; Kocer *et al.*, 2007; Pereira *et al.*, 2009; Pimenta *et al.*, 2012a). Likewise, our research group suggested a possible association between *PRND* gene polymorphisms and ram sperm traits, freezability and consequent fertility (Baptista *et al.*, 2008; Pereira *et al.*, 2009). Therefore, the first objective of this work was to compare the quality and freezability of sperm obtained from rams with different previously identified polymorphisms in codon 26 of *PRND* gene. As abnormal *PRND* gene expression may be involved in human male sterility (Peoc'h *et al.*, 2002), we also investigated if the presence of *PRND* polymorphisms would affect the quality, freezability and Doppel expression of human spermatozoa.

According to Mesquita *et al.* (2010), in the ovine Portuguese breeds, the only polymorphism identified in the *PRND* gene coding region is the synonymous 78G>A substitution in codon 26, coding for Alanine. Although Churra Galega Mirandesa breed has a significantly higher prevalence of heterozygotes GA (25%), when compared to the total population, thus being the eligible breed for the present study, the A allele is globally presented at low frequencies. Due to the rareness of animals with *PRND* AA genotype (Pereira *et al.*, 2009; Mesquita *et al.*, 2010), only one ram from Churra Galega Mirandesa breed was found to have this *PRND* genotype.

Previously, Baptista *et al.* (2008) identified an improved sperm fresh vitality and a decrease of FT sperm head anomalies in samples from rams carrying the *PRND* GA genotype compared to those with the GG one. In the present work, animals classified with GG genotype presented also more (P=0.04) morphological abnormalities (including spermatozoa mid piece defects; P=0.01) than GA or AA rams, but in fresh sperm. However, no differences were observed in sperm vitality (Table 6 and 7) and post-thawed morphology among animals with distinct *PRND* genotypes (Table 9). In what concerns post-swim-up spermatozoa quality parameters, no differences were identified among animals with distinct *PRND* genotypes either (Table 7). Notwithstanding, FT semen from rams carrying the AA genotype significantly (P≤0.04) increased embryo rates when compared with the other two genotypes (Table 10). As in Pereira *et al.* (2009), no differences were identified in embryo production rates

obtained with sperm from GG and GA rams. Nevertheless, in the latter study these authors did not test semen from AA rams. The spermatozoa fertilizing capacity is the best indicator of post-thawed sperm quality (Morris *et al.*, 2001; Valente *et al.*, 2010) which is undoubtedly augmented in our ram presenting the rare *PRND* AA genotype.

Although the identified polymorphism in the ovine *PRND* gene is a synonymous one (G>A), recent studies have indicated that both synonymous and non-synonymous SNPs can influence mRNA stability, processing and maturation, thereby affecting its allelic expression (Komar, 2007). Moreover, synonymous codon substitutions (namely changes from frequent to infrequent codons) may lead to different kinetics of mRNA translation (protein), and thus affect the co-translational folding pathway, yielding a protein with a different final structure and function (Komar, 2007). Besides presenting the higher prevalence of the rare allele A, the Churra Galega Mirandesa breed has the lowest ARR/ARR genotype frequency of our local breeds (Mesquita *et al.*, 2010). Thus the European Union selection program to eradicate scrapie, based only on *PRNP* genotypes (Ehling *et al.*, 2006; Gama *et al.*, 2006), can have unintended consequences on this particular breed, and possible repercussions on reproduction traits and genetic diversity of sheep European breeds. Moreover, previous work from our group (Baptista *et al.*, 2008; Pereira *et al.*, 2009; Mesquita *et al.*, 2010) focused on this synonymous polymorphism located in codon 26 of the ovine *PRND* gene, helped to reinforce the relevance of the Dpl N-terminal region through the established association with the susceptibility to scrapie disease and ram reproductive traits. However, it remains unclear whether this polymorphism could modify structural and/or functional aspects of Doppel, affecting spermatozoa fertilizing capacity as shown in the present study.

In human, our results suggest no relationship between the distribution of *PRND* codon 174 genotypes and male classified according to their fertility status (fertile men, that already have progeny without resorting to medically assisted reproduction, vs infertile, with no progeny) or to fertility treatment at the Center of Medical Reproduction of British Hospital Lisbon XXI (spermogram, IIU and ICSI) or to WHO classification (P>0.05; Table 3), as also described by Peoc'h *et al.* (2002). This polymorphism within the coding sequence of exon 2 (codon 174 of *PRND* gene) is quite frequent among men and it lies at three residues from C-terminal hydrophobic sequence, so according to several authors, it would be unlikely to affect Doppel protein structure and/or function, impairing spermatogenesis and/or sperm motility (Mead *et al.*, 2000; Schröder *et al.*, 2001; Peoc'h *et al.*, 2002). However herein male donors carrying the CC genotype (wild-type) tend to have a higher (P=0.09; Table 12) FT sperm vitality when compared to the other two (CC=42.0 ± 7.63%, CT=30.2 ± 5.62% and TT=18.5 ± 7.24%; Table 12). On the other hand, the 3'UTR polymorphism lies 38 bp 3' to the codon 174 and this two polymorphisms are in linkage (Table 5; Mead *et al.*, 2000). Still, the relationship between the 3'UTR polymorphisms and male reproduction remains unclear (Table 4). Noteworthy, in the analyzed male donors, the *PRND* T allele of 3'UTR polymorphism is linked (P<0.0001) to the *PRND* C allele of codon 174, whereas the

PRND C allele of 3'UTR polymorphism is significantly associated with *PRND* T allele of codon 174 (P=0.001) and differences were identified among their quality parameters evaluations of FT semen (Table 5). Interestingly, after analyzing these male donors according to their linkage between codon 174 and 3'UTR polymorphisms, a five times higher (P<0.001) number of viable spermatozoa after thawing and centrifugation in density gradients were identified in male donors with no linkage between this polymorphisms than in those carrying the linkage (Table 15). As viable spermatozoa are of primordial importance to successfully fertilize the oocyte, these results may have implications on the clinical approach to the infertile men and ART outcomes. However, a total of 29 male donors is not a representative group and further investigation is needed. Moreover, a silent polymorphism was also observed in codon 174 at the third position in only 1/29 individual (Fig. 7d), which also carried the wild-type polymorphism for the regular codon 174. This rare polymorphism was already described by Mead *et al.* (2000), but up to now no correlation with male fertility has been pointed out. Taking altogether, our results indicate that male donors carrying distinct *PRND* genotypes may present differences in the quality and freezability of ejaculates, which could represent an interesting path for future research.

Typically defined as a failure to conceive after a year of regular unprotected intercourse, infertility affects 8% to 16% of reproductive age couples. This condition can be caused by disruptions at various steps of the reproductive process (Medical Advisory Secretariat, 2006). According to Agarwal *et al.* (2014), spermatozoa morphology is probably the most relevant parameter of traditional semen evaluation, providing information of the fertilization potential, having the best indication value of poor semen quality in the laboratory assessment of infertile men. Besides that, our results suggest that fertile male donors have less sperm volume (P=0.03; Table 11), and a tendency to present higher sperm concentration (P=0.08; Table 11); our results from FT samples from fertile male donors also highlight the tendency to present more spermatozoa mid piece abnormalities (P=0.07; Table 14). Although analyzing sperm data from a small population, the differences between fertile and infertile males or males classified according their applied treatments showed a pale positivity. These results are in accordance with the reported growing prevalence of men infertility.

As referred, Doppel is permanently expressed in Sertoli cells, but its expression in the germ cells can vary accordingly to species (Moore *et al.*, 1999; Silverman *et al.*, 2000; Tranulis *et al.*, 2001; Peoc'h *et al.*, 2002; Rondena *et al.*, 2005; Serres *et al.*, 2006; Kocer *et al.*, 2007; Pimenta *et al.*, 2012a). In ovine, Espenes *et al.* (2005) detected Doppel in the seminiferous epithelium in the final stages of spermatogenesis, but not in the ejaculated spermatozoa. On the other hand, bovine and human Doppel was found to be expressed in the ejaculated spermatozoa *(Rondena et al., 2005; Serres et al.*, 2006). Moreover, in human Peoc'h *et al.* (2002) detected this protein on the flagella of mature ejaculated spermatozoa and suggested its involvement in the motility and in active protection of the spermatozoa. In bovine, an intense staining

extending from the neck to the middle piece was detected in ejaculated spermatozoa (Rondena *et al.*, 2005). In our study, using indirect immunofluorescence technique, ovine Doppel could be detected in ejaculated spermatozoa. However, the staining pattern of ovine Doppel is not easy to achieve in spermatozoa, as referred also by Espenes *et al.* (2005). Positive fluorescence in ram sperm head apical region, mid piece and tail could be observed, although the sperm head fluorescence was not always present. Also, as in Pimenta *et al.* (2012b), a nonspecific staining from the FITC-conjugated secondary antibody in sperm mid piece and tail was sometimes identified in ovine spermatozoa, although the brightness intensity was superior in the presence of the produced monoclonal anti-Doppel antibodies, M1 or M2. Nevertheless it was not possible to quantify differences among samples from rams classified according to their *PRND* genotypes. This might suggest an expression level in ovine smaller than the detection limit of this method (as previously referred by Espenes *et al.* (2005)) or a different biological behavior of ovine Doppel protein related to different location of both soluble and insoluble forms in the male germ cells, demanding further studies. In human spermatozoa, the positive staining in sperm head, mid piece and tail was also detected in the negative controls. Accordingly to WHO (World Health Organization, 2010), male donors with more than 4% of spermatozoa with normal morphology are considered fertile, a fact that point out the elevated prevalence of abnormal human spermatozoa. Thereby, the low presence of morphologic normal sperm might dilute and/or mask the real human Doppel localization. Thus, our produced antibodies could not specifically detect human Doppel expression in ejaculated spermatozoa.

In ovine testis, Doppel protein appears to carry two N-glycans, but apparently lacks O-glycans (Espenes *et al.*, 2005). After being synthesized in the endoplasmic reticulum, the Doppel polypeptide is processed at its C- and N- terminus and is then exposed to the cell membrane (Uelhoff *et al.*, 2005). Recent reports from Pimenta *et al.* (2013b) suggest that the N-terminal signal peptide adopts, immediately upon synthesis and while connected to the ribosome in the aqueous environment, an essentially random structure. Interaction with the SRP54M monomer then takes place. Upon binding, the signal peptide may then stabilize into a dynamic α-helical conformation, maintained within the hydrophobic environment of SRP54M, thus promoting its later binding to the endoplasmic reticulum translocator receptor. Conversely, observations in cells and tissues suggest that Doppel may also exist as an intracellular form devoid of the GPI anchor (Peoc'h *et al.*, 2002; Cordier-Dirikoc *et al.*, 2008). The existence of different Doppel forms either associated to the cell membrane via a GPI anchor, or in the intra- or extra-cellular spaces was proposed by Peoc'h and Laplanche (2006). Thus, a soluble form of Doppel protein was used during ovine sperm capacitation improving their motility, viability and fertilization rate (Pimenta *et al.*, 2012a). The distribution as well as the function of both forms of Doppel protein may be species specific or related to spermatozoa quality and thus influencing their accessibility to antibodies and consequently the staining patterns. Also, Dpl contains a basic sequence at the N-terminal (25-30) similar to the basic sequence (residues 23-28) found at the N-terminal

of PrP^C (Papadopoulos *et al.*, 2006) that resembles a nuclear localization sequence which in term may contribute to a certain degree of immunofluorescence antigen masking during nuclear uptake (Biverstahl *et al.*, 2004; Pimenta *et al.*, 2013b).

Flow cytometry experiments were conducted with human and bovine mature ejaculated spermatozoa to successfully identify Doppel expression (Peoc'h *et al.*, 2002; Rondena *et al.*, 2005). Moreover, these last authors suggested an extensive application of this technique to animals with reduced fertility as a new parameter to consider during infertility studies. Therefore, in the present study flow cytometry was performed for two purposes: to quantify and differentiate ovine Doppel expression from animals with different *PRND* genotypes using both fresh and FT semen, and to discriminate which of the two produced anti-Doppel monoclonal antibodies (M1 and M2) was more specific for ovine Doppel detection. Regardless of the *PRND* genotype, we report here for the first time the differences of ovine Doppel detection between fresh (Fig. 14a) and FT (Fig. 14b) semen by using both M1 and M2 antibodies ($P<0.03$ and $P<0.05$, respectively; Table 16). These observations strongly suggest that Doppel protein may suffer some conformational and biochemical alterations during the freezing process, and are also consistent with the findings of Pimenta *et al.* (2012b), where a new developed anti-ovine Prt polyclonal antibody was unable to detect caprine Prt only due to subtle differences between ovine and goat Prt predicted tertiary structures.

The use of FT semen for artificial insemination in sheep has not yet proved to be satisfactory. Indeed, artificial insemination is carried out mainly with chilled semen because of the low fertility results obtained when using FT semen in this species (Anel *et al.*, 2005; Barbas *et al.*, 2013b). The sperm plasma membrane is the primary site of damage induced by cryopreservation, but DNA damage and instability were also reported, effectively reducing the fertilization efficiency of the FT semen (Bailey *et al.*, 2000; Peris *et al.*, 2007; Romão *et al.*, 2013). Nevertheless, an individual variability among males for the susceptibility to the process of cryopreservation has been widely established (Hoffmann *et al.*, 2011; Barbas *et al.*, 2013a). Likewise, our results showed a decrease in Doppel expression after the ovine spermatozoa FT process (Table 16; Fig. 14). Additionally, a greater Doppel mRNA abundance was detected in FT semen of bulls with high sire conception rate by Kasimanickam *et al.* (2012). Altogether, these results suggest that Doppel protein expression could be used to build a diagnostic tool to select males for improved cryoresistance and reproductive performance, or on the contrary, to identify the sub/infertile ones.

5. CONCLUSION

In conclusion, presented data confirm that the identified polymorphisms in codon 26 of ovine *PRND* gene are important for an improved semen cryoresistance and embryo production. Specifically the AA genotype should be actively selected in rams and *PRND* used as a candidate gene in breeding programs strategy. Three *PRND* polymorphisms were found for the first time in the human male Portuguese population. A linkage between codon 174 and 3'UTR polymorphisms of *PRND* gene was detected. Despite the small sample size, an enhanced viability after thawing and centrifugation in density gradients of post-thawed spermatozoa was observed in human male donors presenting CT genotype of codon 174 and TT genotype of 3'UTR polymorphism. The relationship between these polymorphisms and male reproduction demands further studies.

Furthermore the identification of Doppel protein in ovine ejaculated spermatozoa and its decrease expression after the cryopreservation process strongly suggests an important physiological function in male fertility, opening news insights to the possibility of male selection with improved cryoresistance and reproductive performance or towards the development of clinical treatment of infertility.

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7. ANNEXES

7.1. Annex I – culture media and solutions

The following media are currently used in andrology and embryology laboratories in Animal Reproduction and Genetic Improvement Department at INIAV (Santarém, Portugal).

Table 17 – MRF-SSCP denaturing solution

Reagent	Company	Reference	Concentration
Formamide	Invitrogen	15515-026	95% (w/v)
NaOH	Sigma-Aldrich	S5881	10 mM
Xylene cyanol	Sigma-Aldrich	X4126	0.05% (w/v)
Bromophenol blue	Sigma-Aldrich	B0126	0.05% (w/v)

Table 18 – Cryoprotective medium

Reagent	Company	Reference	Concentration	Amount
Trizma Base	Sigma-Aldrich	T1503	0.36 M	2.1805 g
Citric Acid	Merck	1.00244.1000	0.11 M	1.1880 g
Glucose	Sigma-Aldrich	G6152	0.03 M	0.3000 g
Egg yolk	--	--	15% (v/v)	8.0 mL
Glycerol	Merck	1.04091.1000	6.5% (v/v)	3.5 mL
Antibiotics (sorocimicine)	Laboratório Sorológico	--	0.1% (w/v)	0.0500 g
H_2O	--	--	--	38.0 mL

Table 19 – Ovine swim-up and sperm capacitation medium

Reagent	Company	Reference	Concentration	Amount
NaCl	Sigma-Aldrich	S5886	0.12 M	0.3580 g
KCl	Sigma-Aldrich	P9333	4 mM	0.0150 g
$NaHCO_3$	Merck	1.06329.0500	4 mM	0.0169 g
NaH_2PO_4 H_2O	Sigma-Aldrich	S9638	0.7 mM	0.0050 g
$MgCl_2$ $6H_2O$	Sigma-Aldrich	M0250	0.5 mM	0.0053 g
$CaCl_2$ $2H_2O$	Sigma-Aldrich	C5080	2 mM	0.0165 g
Hepes	Sigma-Aldrich	H3375	0.01 M	0.1200 g
Sodium pyruvate	Sigma-Aldrich	P4562	9 mM	0.0550 g
BSA	Sigma-Aldrich	A7888	0.3% (w/v)	0.1500 g
Glucose	Sigma-Aldrich	G6152	0.01 M	0.1250 g
Sheep superovulated oestrus serum [1]	--	--	20% (v/v)	8 mL
H_2O	Sigma-Aldrich	W1503	--	40 mL

[1] See sheep superovulated oestrus serum protocol.

Table 20 – CTC buffer solution

Reagents	Company	Reference	Concentration	Amount
NaCl	Sigma-Aldrich	S5886	0.04 M	0.240 g
Tris Base	Sigma-Aldrich	T1503	0.06 M	0.760 g
H_2O	--	--	--	100 mL

Table 21 – CTC staining solution

Reagents	Company	Reference	Concentration	Amount
Chlortetracycline	Sigma-Aldrich	26430	0.8 mM	0.0020 g
L-cysteine	Merck	1.02838.0025	7 mM	0.0044 g
CTC buffer solution	--	--	--	5 mL

Table 22 – JC-1 incubation medium (adapted from Gamboa *et al*. (2010)**)**

Reagents	Company	Reference	Concentration	Amount
Hank's Balanced Salt solution	Sigma-Aldrich	H6648	--	10 mL
BSA	Sigma-Aldrich	A7888	1% (w/v)	0.1 g
Hepes	Sigma-Aldrich	H3375	19.5 mM	0.0466 g
JC-1 in DMSO	Sigma-Aldrich	T4069	8 nM	1 µL

Table 23 – Synthetic oviduct fluid (SOF) medium

Reagents	Company	Reference	Concentration	Amount
NaCl	Sigma-Aldrich	S5886	0.11 M	0.3147 g
KCl	Sigma-Aldrich	P9333	7 mM	0.0267 g
KH_2PO_4	Merck	1.04873.0250	1 mM	0.0081 g
$CaCl_2$ $2H_2O$	Sigma-Aldrich	C5080	2 mM	0.0126 g
$MgCl_2$ $6H_2O$	Sigma-Aldrich	M0250	0.5 mM	0.0049 g
$NaHCO_3$	Merck	1.06329.0500	0.02 M	0.105 g
Sodium lactate	Merck	1.06522.2500	3 mM	14.1 µL
Sodium pyruvate	Sigma-Aldrich	P4562	0.3 mM	0.0017 g
Phenol red	Sigma-Aldrich	P3532	4 µM	0.000065 g
L-Glutathione	Sigma-Aldrich	G6013	0.1 mM	0.00155 g
L-Glutamine	Sigma-Aldrich	G8540	0.86 mM	0.25 mL
BME amino acids solution	Sigma-Aldrich	B6766	2%	1 mL
MEM non-essential amino acids solution	Sigma-Aldrich	M7145	1%	0.5 mL
H_2O	Sigma-Aldrich	W1503	--	50 mL

Table 24 – Oocyte maturation medium

Reagents	Company	Reference	Concentration	Amount
Medium 199	Sigma-Aldrich	M4530	--	5 mL
Gentamicine	Sigma-Aldrich	G1272	4 $\mu L mL^{-1}$	20 µL
Epidermal Growth Factor	Sigma-Aldrich	E4127	10 $ng mL^{-1}$	50 µL
Cysteamine	Sigma-Aldrich	M9768	0.08 mM	50 µL
β-Estradiol	Sigma-Aldrich	E4389	10 $\mu L mL^{-1}$	50 µL

Table 25 – In vitro fertilization (IVF) medium

Reagents	Laboratory	Reference	Concentration	Amount
SOF medium	--	--	--	9 mL
Sheep superovulated oestrus serum [1]	--	--	10% (v/v)	1 mL
Gentamicine	Sigma-Aldrich	G1272	4 $\mu L mL^{-1}$	40 µL

[1] See sheep superovulated oestrus serum protocol.

Sheep superovulated oestrus serum protocol

A sponge impregnated with progesterone was placed into ewe vagina (Day 0) and kept for 9 days. At Day 8, 24 hours before sponge removal, 150 µg of PGF2α plus 1500 IU of eCG were administrated to the ewe. Blood samples were collected 24-30 hs after sponge removal. Blood samples were placed at 4^{o}C and serum manipulated 12-18 hours after collection.

Table 26 – Zygote transfer medium

Reagents	Company	Reference	Concentration	Amount
SOF medium	--	--	--	12.5 mL
BSA	Sigma-Aldrich	A7888	0.6%	0.075 g

Table 27 – *In vitro* embryo culture medium

Reagents	Company	Reference	Concentration	Amount
SOF medium	--	--	--	9 mL
BSA	Sigma-Aldrich	A7888	0.6%	0.060 g
Foetal bovine serum	Sigma-Aldrich	F9665	10%	1 mL

7.2. Annex II – oral presentations

6th Veterinary Sciences Congress: Praxis and Future
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Sperm capacitation status in fresh and cryopreserved ovine semen and its effect on in vitro embryo production

Ferreira LM[1], García-Herreros M[2], Marques CC[1], Barbas JP[1], Baptista MC[1], Horta AEM[1], Pereira RMLN[1,3]

[1]Unidade de Biotecnologia e Recursos Genéticos, Instituto Nacional de Investigação Agrária e Veterinária, Quinta da Fonte Boa, 2005-048 Vale de Santarém, Portugal.
[2] Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos (FZEA), Universidade de São Paulo, Brasil.
[3]Escola Universitária Vasco da Gama, Coimbra, Portugal.

Ram semen cryopreservation causes functional damage to sperm which may impair its fertility. Capacitation status could be associated to sperm lifespan, and therefore, may be linked to the success during fertilization process. Nevertheless, there are different sperm properties such as biophysical changes that may be modified during the freezing-thawing process, contributing to the lower fertility of thawed semen. In this study we aim to identify the capacitation status and morphometrical alterations of both fresh and frozen-thawed sperm and its influence on fertilization ability and embryo production. Ejaculates from four rams were equally divided in two aliquots and used as fresh (Fsh) and cryopreserved (FT) samples for IVF process. Concentration, motility, viability, and sperm head morphometrical characteristics were analysed for Fsh and FT samples. Chlortetracycline staining (CTC) was performed in Fsh samples after collection, after 2 to 6 hours of incubation at room temperature, and after swim-up; and in FT samples after thawing, and after swim-up. Three distinctive sperm populations were identified: non-capacitated (F), capacitated with intact acrosome (B), and acrosome reacted (AR). Mature cumulus-oocyte complexes were inseminated with Fsh or FT sperm samples after swim-up selection process. Eighteen hours pi, presumptive zygotes were cultured in synthetic oviductal fluid (SOF) enriched with aminoacids and bovine serum albumin (BSA) at 38.5 ºC, under 5% O_2, 5% CO_2 and 90% N_2 until 2-to 8-cell embryo stage. After cleavage rate assessment, embryo development was carried out to blastocyst stage in SOF+BSA medium with 10% FCS. Our preliminary results showed that initial, pre- and post-swim-up motility, post-swim-up concentration and vigour were superior in Fsh semen compared to FT samples ($P<0.001$). Before swim-up selection, Fsh sperm showed a higher F and a lower AR percentage than FT sperm ($P<0.05$). After swim-up, the percentage of sperm showing B pattern was higher in Fsh compared to FT samples ($P<0.05$). Regarding sperm head morphometry, dimensional (Area, Perimeter, Length and Width) and shape parameters (Ellipticity, Elongation and Rugosity) changed drastically after cryopreservation process ($P<0.001$). Embryo production rates were higher after insemination with Fsh semen ($P<0.05$). Our results indicate there is an important effect

of the freezing-thawing process on ram sperm motility, vigour, head morphometry and capacitation status with repercussion in fertilization and embryo production.

Cryotolerance of bovine oocytes: Is membrane permeability to water and cryoprotectants really important?

Matos JE[1], Marques CC[2], Ferreira LM[2], Baptista MC[2], Horta AEM[2], Moura T[1], Soveral G[1], Pereira RMLN[2,3]

[1] Faculdade de Farmácia. Universidade de Lisboa, Lisboa, Portugal.
[2] Unidade de Biotecnologia e Recursos Genéticos, Instituto Nacional de Investigação Agrária e Veterinária, Quinta da Fonte Boa, Vale de Santarém, Portugal.
[3] Escola Universitária Vasco da Gama, Coimbra, Portugal.

Oocyte cryopreservation is increasingly in demand due to the need for preserving gametes of both humans and animals. This technique requires several steps during which cells must withstand extreme anisosmotic conditions and their associated cell volume fluxes. These volume excursions place a cumulative osmotic stress on the cell. Therefore the permeability of the plasma membrane to water and cryoprotectants is one of the important cryobiological properties affecting the survival of an oocyte after vitrification. Our goal was to determine and manipulate bovine oocyte membrane permeability to water and two cryoprotectants, ethylene glycol (EG) and dimethyl sulfoxide (DMSO), often used in oocyte cryopreservation protocols. Moreover their effects on oocyte post-thawed developmental competence were further determined. Abattoir derived bovine oocytes were cultured for 22- 24h with 100 µM trans10 cis12 conjugated linoleic isomer (CLA) or without supplementation (CLA and control groups). Fresh mature oocytes were immobilized in glass slides coated, observed in an inverted microscope and the dynamic of oocyte volume changes quantified. To investigate oocyte permeability to water and cryoprotectants, two solutions containing 200 mM sucrose or 10% DMSO and 10% EG were used. Mature oocytes from both groups were vitrified using cryotop and warmed (4 sessions) prior to *in vitro* fertilization. Embryo culture proceeded in SOF supplemented medium. Results showed a significant CLA effect ($p<0.001$) on the osmotic water permeability: permeability in control oocytes was significantly higher (0.014 ± 0.002 cm.s^{-1}; n=11) than in oocytes matured with CLA (0.010 ± 0.002 cm.s^{-1}; n=11). Similar effect was observed on the cryoprotectants permeability ($p<0.001$). Moreover CLA improved ($p<0.05$) the developmental competence of frozen-thawed oocytes. In conclusion, CLA presence during maturation slowdown oocyte membrane permeability to water, DMSO and EG, although improving subsequent cryosurvival. Therefore a dichotomy between the membrane permeability to cryoprotectants and cryotolerance was identified in bovine oocytes.